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# MYOCARDIAL NECROSIS AND FIBROSIS RESULTING FROM THE ADMINISTRATION OF MASSIVE DOSES OF A CARDIAC GLYCOSIDE[1]

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Most of the toxic symptoms produced by the cardiac glycosides have been exhaustively studied, but the pathological changes resulting from the administration of massive doses of these drugs are not so well known. Essex, Herrick and Visscher (1) have shown that lanatosides A, B, and C in doses of from 3 to 25% of the minimum lethal dose do not reduce the coronary blood flow in unanaesthetized dogs. Larger doses, however, were found to reduce the coronary blood flow (2). Bauer (3), Hu (4), Büchner (5) and Lindner (6) demonstrated that digitoxin, when given in doses approximating one-third of a cat unit (Hatcher-Brody) per kilo of body weight over a period of from 5 to 30 days, produces degenerative changes in the heart muscles of dogs. The writer has previously reported the production of necrosis and fibrosis in the heart muscles of dogs following daily intravenous administration of lanatoside C (7). Weese and Dieckhoff (8) saw these changes in cats eight days after the administration of single doses of 0.2 mg. of digitoxin per kilo intravenously. All these investigators describe findings suggestive of restriction of coronary blood supply, although no gross or microscopic changes could be demonstrated in the vessels themselves. In some sections the muscle fibres showed loss of striation, branching and pyknotic nuclei, accompanied by mononuclear and polynuclear cell infiltration. Elsewhere, frank necrotic and infarcted areas appeared in the sections. Finally, when the animals survived the acute experiments, fibrosis was sometimes very marked.

Lendle (9) studied the effects of atropine and vagotomy upon the Hatcher cat dosage of strophanthus. His normal animals were killed by 0.08 to 0.12 mg. per kilo. When the cats were throughly atropinized, however, the fatal dosage was significantly increased (0.12 to 0.147 mg. per kilo). The tolerance of cats which had previously been vagotomized was lower than that of both the above groups—0.056 to 0.144 mg. per kilo, with only one animal requiring more than 0.10 mg. Hahn (10) showed that vagotomized cats were even more sensitive to cardiac glycosides than Lendle's data indicated.

Despite the fact that the doses employed by these investigators were far in excess of the largest single dose ever given clinically, it seemed worthwhile to conduct comparable experiments with lanatoside C (11) and digitalis purpurea over a wider range of dosage. The purpose of this study was to find out if the oral administration of digitalis folia and lanatoside C in amounts comparable to those given clinically would produce changes in the heart muscles of dogs; to determine, if possible, the smallest daily intravenous dose of lanatoside C which would cause degenerative myocardial damage; to correlate electrocardiographic changes with clinical symptoms and morphological changes in the heart muscle; and to determine what effect the giving of atropine might have upon the toxic symptoms and survival time of dogs receiving large daily doses of lanatoside C intravenously.

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[1] This paper comprises part of a thesis submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine. Part of this material was presented at the meetings of the American Federation for Clinical Research in Minneapolis, April, 1941.

PROCEDURE. Four series of dogs were studied. In the first series of 9 dogs, 5 animals received lanatoside C and 4 digitalis purpurea (U.S.P.X.). An initial dose of 1/10 of a cat unit of lanatoside C or tincture of digitalis per 10 pounds of body weight was given by vein. Thereafter doses of from ½ to 5 cat units of lanatoside C or digitalis folia were administered orally once daily in tablet form in hamburger balls. Each tablet of lanatoside C contained 0.25 mg., equivalent to one Hatcher-Brody cat unit; each 1½ grain tablet of digitalis purpurea contained one cat unit.

The second group of 8 dogs was given daily intravenous injections of lanatoside C[3] varying from 1/20 to ¾ of a cat unit per kilo of body weight.

A third series of 9 dogs was given daily intravenous injections of from ⅓ to ¾ of a cat unit of lanatoside C per kilo of body weight. Serial electrocardiograms were made throughout this experiment.

The fourth group of 6 dogs received daily intravenous injections of from 3/10 to ½ a cat unit of lanatoside C, had serial electrocardiograms taken, and in addition were given subcutaneous injections of 1.5 mg. of atropine sulfate twice daily.

The dogs were killed (or died) at varying intervals of time and complete post-mortem studies were made. Thirty sections were taken from each heart and routine slides were made of the other organs.

EFFECT OF PROLONGED DAILY ADMINISTRATION OF ORAL LANATOSIDE C OR DIGITALIS PURPUREA (LEAF). Seven of the 9 dogs in the first group (table 1) took up to 2 cat units (0.044 to 0.200 cat units per kilo) either of digitalis folia or of lanatoside C daily for 26 to 368 days without any signs of toxicity. Since these animals weighed about 30 pounds each, these dosages are comparable to 2½ to 10 cat units per day in man, equilibrating relative amounts on a basis of 30 to 150 pounds.

Four of the dogs were sacrificed after 368 days and one after 208 days. There were no abnormal gross autopsy findings and the 30 microscopic sections taken from each heart appeared normal in all respects. Two of the dogs died of pneumonia on the 26th and 50th days of the experiment, 8 and 12 hours before autopsy was done. No abnormal histology was found except in the lungs.

The last two dogs in this group received 5 tablets of the chosen drug daily for 85 days, one taking 0.370 cat units of lanatoside C per kilo of weight daily and the other 0.430 cat units of digitalis purpurea. Although these dogs exhibited progressive anorexia, lost weight and appeared weak, there were no other signs of digitalis toxicity. Autopsies of these animals revealed no gross or microscopic evidence of abnormalities. The coronary vessels were smooth, thin, elastic and patent throughout. The heart muscle fibrils stained well with hemotoxylin-eosin and exhibited normal structure.

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[3] Each cc. of solution contained 0.20 mg. of the drug.

These results demonstrate the wide margin of tolerance of dogs to folia digitalis and lanatoside C when these drugs are given by mouth over a long period of time in daily doses of from 0.044 cat units to 0.430 cat units per kilo and indicate that therapeutic doses of digitalis will not produce morphological changes in the heart muscle.

TABLE 1

*The effect of prolonged daily administration of oral lanatoside C and digitalis purpurea (leaf)*

| DOG NO. | NO. OF TABLETS GIVEN DAILY | DOSAGE PER KILO | DURATION OF EXPERIMENT | SYMPTOMS NOTED DURING EXPERIMENTS | PATHOLOGICAL FINDINGS |
|---|---|---|---|---|---|
| | | *cat units* | *days* | | |
| 1 | ½ | 0.044 | 368 | None | None |
| 2 | 1 | 0.065 | 368 | None | None |
| 3 | 2 | 0 134 | 368 | None | None |
| 4 | 1 | 0.060 | 368 | None | None |
| 5* | 1 | 0 080 | 50 (D) | Died, pneumonia | ? |
| 6* | 2 | 0 200 | 208 | None | None |
| 7* | 1 | 0.099 | 26 (D) | Died, pneumonia | ? |
| 8 | 5 | 0.370 | 85 | Anorexia, wt. loss, muscular weakness | None |
| 9* | 5 | 0.430 | 88 | Anorexia, wt. loss, muscular weakness | None |

* *Starred numbers indicate animals given digitalis purpurea. (D) represents animals dying 8 to 12 hours before autopsy.*

TABLE 2

*The effect of daily intravenous injections of lanatoside C*

| DOG NO. | DAILY DOSAGE | DOSAGE PER KILO | DURATION OF EXPERIMENT | SYMPTOMS NOTED DURING EXPERIMENTS | HEART MUSCLE PATHOLOGY |
|---|---|---|---|---|---|
| | *cc.* | *cat units* | *days* | | |
| 1-v | 1.0 | 0 055 | 33 | None | None |
| 2-v | 1.0 | 0.071 | 33 | Anorexia, wt. loss, sialorrhea, muscular weakness | None |
| 3-v | 1.5 | 0.106 | 33 | Same as 2-v | None |
| 4-v | 3.22 | 0 166 | 32 | Same as 2-v, with emesis | None |
| 5-v | 5.0 | 0 250 | 32 | Above, accentuated, dyspnea after last 4 injections | None |
| 6-v | 6.1 | 0 333 | 25 (D) | Same as 5-v | ? |
| 7-v | 10 8 | 0.500 | 7 (D) | Same as 5-v | ? |
| 8-v | 12.4 | 0 750 | 4 (D) | Same as 5-v | ? |

(D) represents animals dying 6 to 12 hours before autopsy.

Effect of daily intravenous injections of lanatoside C. The 8 dogs in the second group were given daily intravenous injections of lanatoside C (table 2). The dosage varied from $\frac{1}{20}$ to $\frac{3}{4}$ of a cat unit per kilo of body weight. *Five* of the animals were sacrificed after 32 and 33 days, the other 3 died unexpectedly.

The coronary vessels of the hearts of all the dogs examined post-mortem were smooth and elastic with widely patent lumens. Sub-endocardial hemorrhages were noted in one of the hearts which was microscopically normal and in 3 of the hearts showing questionable cellular damage. There were no other significant gross autopsy findings.

Microscopic studies showed no abnormalities among the first 5 dogs' hearts. Unfortunately, the dogs receiving $\frac{1}{3}$, $\frac{1}{2}$, and $\frac{3}{4}$ of a cat unit of lanatoside C per kilo died during the night and post-mortem changes in their hearts were too extensive to permit accurate conclusions. However, areas of infarction with mononuclear infiltration and even some connective tissue proliferation were noted. It seems unlikely that these alterations in the myocardium could have occurred in the 8 to 12 hours preceding autopsy.

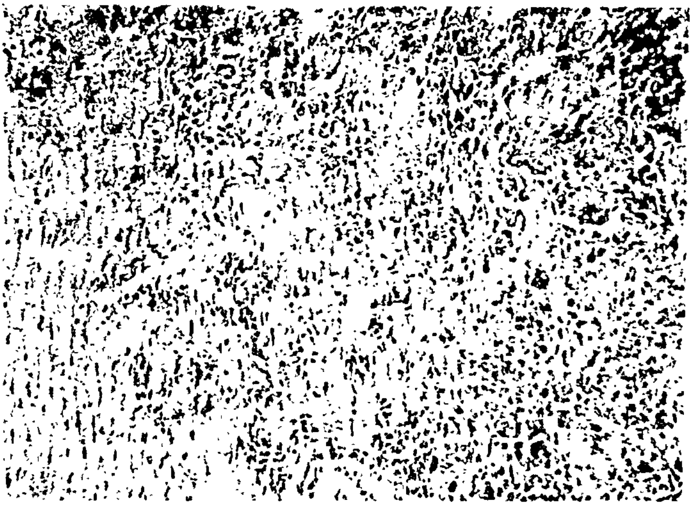

FIG. 1. PHOTOMICROGRAPH OF HEART MUSCLE OBTAINED FROM DOG 7-E, SHOWING AN AREA OF ACUTE MYOCARDIAL INFARCTION

Note loss of striation of muscle fibres and cellular infiltration. Hemotoxylin-eosin stain was used

The third series of 9 dogs was given daily intravenous injections of lanatoside C in amounts above $\frac{1}{5}$ of a cat unit per kilo of weight (table 3). Daily electrocardiograms were taken. Three of these dogs died 4 to 8 hours before autopsy and the cellular changes seen in their hearts were disregarded. The dogs taking $\frac{1}{4}$, $\frac{1}{3}$, and $\frac{2}{5}$ of a cat unit per kilo lived 8 days; there were no significant microscopic changes in the muscles of their hearts. The hearts of the last 3 dogs in this group exhibited diffuse and widespread cellular damage (figs. 1 and 2). Numerous small infarctions containing a few mononuclear cells and very occasional polymorphs were noted in most of the sections. Elsewhere there was marked proliferation of young connective tissue replacing some of the necrotic muscle.

An unusual atrophy of cardiac muscle without necrosis was seen in two of the sections. Occasional muscle fibers had lost their striations and were frayed at the ends. Muscular atrophy, loss of striation and fraying of muscle fibers, and infarction appear to be the primary pathological changes resulting from massive daily doses of the glycoside; the mild mononuclear and polymorphonuclear exudate and the connective tissue reactions probably represent secondary changes in response to the primary damage.

All dogs in this group showed signs of starvation associated with their marked anorexia and persistent vomiting. One had persistent diarrhea in addition to the other toxic reactions noted in table 3. Besides the microscopic changes resulting from the administration of lanatoside C, the heart muscles showed other changes suggestive of vitamin B deficiency.

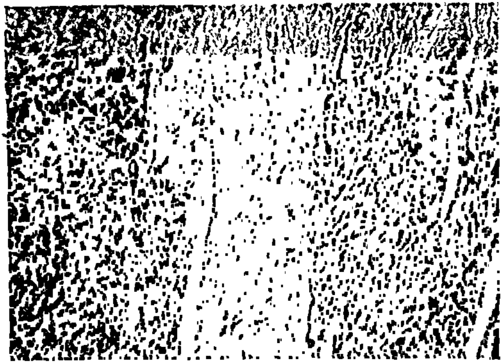

FIG 2 PHOTOMICROGRAPH OF HEART MUSCLE OBTAINED FROM DOG 5-E, SHOWING COMPLETE REPLACEMENT OF LARGE AREAS OF THE HEART MUSCLE BY FIBROTIC TISSUE
Note intense cellular reaction. Hemotoxylin-eosin stain was used

Wenckebach (12) and Weiss and Wilkins (13) saw hydropic swelling of muscle cells, fraying of fibers, loss of striation and nuclear changes in the hearts of patients dying with beri-beri. These same changes were observed in several of our most severely starved dogs.

All the dogs in the second and third groups (tables 2 and 3) exhibited the same symptoms in varying degree, depending upon the amount of lanatoside C administered. Those receiving more than 5 cc. daily developed severe anorexia, emesis and marked sialorrhea and were at times severely dyspneic. The dyspnea lasted from 4 to 45 minutes after injection of the drug and was accompanied by a tachycardia or bradycardia and, in one instance, by a venous pressure of 15 cm. of water. The significance of generalized muscular weakness as an apparent toxic effect of cardiac glycosides both in dogs and in man has been reporte elsewhere (14).

The mechanism of the production of necrosis, fibrosis and actual atrophy o the myocardium can at present only be assumed. Weese states that large dose of digitalis bodies produce constriction of the coronary vessels and Gilbert an Fenn (15) think that the coronary sinus flow is reduced by these drugs. A previously stated, Essex and Visscher found that lanatosides A, B, and C in dose of from 3 to 25% of the minimum lethal dose do not reduce the coronary flow

*The protective action of atropine.* Anorexia, emesis and sialorrhea suggeste vagus hyperactivity, so the 4th group of 6 animals was given 1.5 mg. of atropin sulfate twice daily during the period of glycoside administration. Table summarizes these experiments. Sialorrhea was inhibited, but all the othe symptoms were present although somewhat diminished. It is worth notin that 4 animals in this group were given nearly one-half a cat unit of lanatoside C per kilo daily, but lived almost three times as long as the animals receiving a similar dosage of the glycoside with no atropine (table 3). Three of these dogs died four to six hours before autopsy. One of the remaining 3 had 2 small areas of connective tissue replacement of heart muscle; the other 2 had normal heart muscles.

TABLE 3

*The effect of daily intravenous injections of lanatoside C correlated with electrocardiograph*

| DOG NO. | DAILY DOSAGE | DOSAGE PER KILO | DURATION OF EXPERIMENT | SYMPTOMS NOTED DURING EXPERIMENTS | EKG CHANGES | HEART MUSCLE PATHOLOGY |
|---|---|---|---|---|---|---|
| | *cc.* | *cat units* | *days* | | | |
| 1-E | 5.0 | 0.25 | 8 | Anorexia, wt. loss, emesis, muscular weakness, sialorrhea, dyspnea | Present | None |
| 2-E | 6.9 | 0.33 | 8 | Same as 1-E | Present | None |
| 3-E | 12.7 | 0.50 | 4 (D) | Same as 1-E | Present | ? |
| 4-E | 12.6 | 0.75 | 4 (D) | Same as 1-E | Present | ? |
| 5-E | 3.0 | 0.20 | 60 | Same as 1-E, except for absence of dyspnea | Present | Present |
| 6-E | 6.0 | 0.25 | 22 | Same as 5-E | Present | Present |
| 7-E | 8.0 | 0.33 | 7 | Same as 1-E | Present | Present |
| 8-E | 13.6 | 0.50 | 7 (D) | Same as 1-E | Present | ? |
| 9-E | 9.6 | 0.40 | 8 | Same as 1-E | Present | None |

(D) represents animals dying 6 to 12 hours before autopsy.

Our data on the protective effect of atropine against daily intravenous injections of more than 0.2 cat units of lanatoside C per kilo of dog appear to substantiate the acute experimental data reported by Lendle (9) and Hahn (10).

*Electrocardiographic changes produced by lanatoside C.* Electrocardiograms were made at frequent intervals on 15 of the dogs receiving large daily intravenous doses of lanatoside C. Serial tracings in every instance showed, at some time during the experiment, the effects of the drug. The first change was usually seen as an alteration in the shape of the T wave or a slight lengthening of the PR interval, although these effects were not always persistent or progressive in any one animal. Alterations in the ST interval were prominent, varying from slight depression to depression with coving. In no instance, however, was there reciprocal elevation and depression of the ST interval in leads I and III. (T wave and ST changes in the electrocardiogram of the dog may occur spontaneously.) Extrasystoles, dropped beats and variable degrees of heart block were observed, the latter and the marked ST changes occurring after 2 to 5 doses of lanatoside C had been given intravenously.

Extreme electrocardiographic changes occurred in 4 of the 15 dogs after they had received 6 to 8 injections of from 0.2 to 0.35 cat units of lanatoside C per kilo of weight. The myocardium of only one of these animals showed degenerative microscopic changes. Figure 3 is a photograph of a series of tracings obtained from one of the animals and is characteristic, illustrating T wave changes, 2:1 heart block, alternating bundle branch block and ventricular tachycardia. Bundle branch and complete heart block were seen in the electrocardiograms of 2 of the 4 dogs. In almost every instance, dogs wth ventricular tachycardia following the intravenous injection of lanatoside C were markedly dyspneic for 10 to 45 minutes, exhibited great weakness, and in one animal the venous pressure rose to 15 cm. of water. Frequently with the subsidence of the dyspnea, the ventricular tachycardia would disappear. No apparent electrocardiographic changes resulted from the administration of atropine, nor did the atropine modify the changes produced by lanatoside C.

TABLE 4

*The effect of daily injections of lanatoside C and of 1.5 mg. of atropine sulfate twice daily, correlated with electrocardiography*

| DOG NO | DAILY DOSAGE | DOSAGE PER KILO | DURATION OF EXPERIMENT | SYMPTOMS NOTED DURING EXPERIMENTS | EKG CHANGES | HEART MUSCLE PATHOLOGY |
|---|---|---|---|---|---|---|
| | *cc.* | *cat units* | *days* | | | |
| 10-E | 9.0 | 0.46 | 21 | Anorexia, wt. loss, muscle weakness, dyspnea | Present | None |
| 11-E | 8.0 | 0 57 | 16 | Same | Present | Slight |
| 12-E | 5.0 | 0.30 | 23 (D) | Same | Present | ? |
| 13-E | 4.0 | 0.24 | 15 (D) | Same | Present | ? |
| 14-E | 6.0 | 0 42 | 15 (D) | Same | Present | ? |
| 15-E | 7.0 | 0.50 | 15 | Same | Present | None |

(D) represents animals dying 4 to 10 hours before autopsy.

Levine and Cunningham (16) noted electrocardiographic changes in anaesthetized cats given increasing amounts of cardiac glycosides by vein. Extrasystoles appeared after 48% of the fatal dose had been given, auriculoventricular dissociation after 70%, and total block when 80% of the fatal dose had been injected.

The appearance of ventricular tachycardia with alternating bundle branch

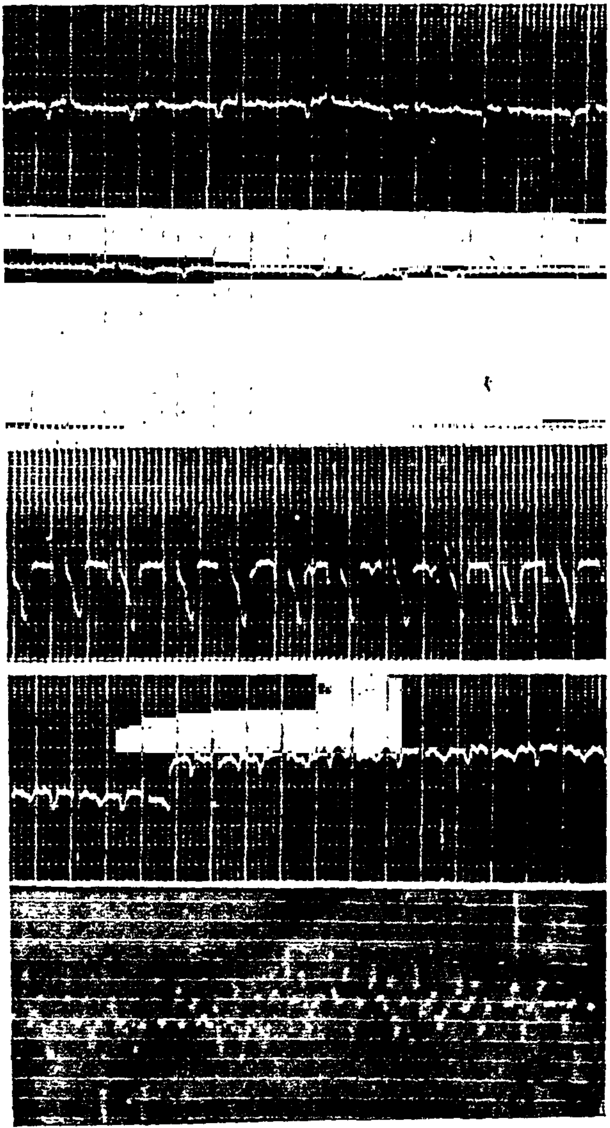

FIG. 3. SERIES OF ELECTROCARDIOGRAMS TAKEN ON DOG 10-E

S/2. Tracing obtained before administration of lanatoside C. It appears somewhat abnormal because the animal was lying on its left side
S/7. After 6 daily injections of 0.46 cat units of lanatoside C per kilo of weight, 2:1 heart block has developed.
S/9. Ventricular tachycardia appeared shortly after the injection of lanatoside C.
S/12. Tracing shows depression and coving of the ST interval.
Tracing obtained shortly after injection of lanatoside C, about one hour before the

block resulting from the experimental administration of cardiac glycosides has not been previously described. Howard (17) reported the case history of a patient who developed alternating bundle branch block following excessive intake of folia digitalis.

The relation between the amount of lanatoside C which can be taken without toxic symptoms and the amount which produces myocardial damage when the drug is administered by vein is suggested by the data obtained on dog 1-v and on dogs 5, 6, 7, and 11-E (tables 3 and 4). Dog 1-v (table 2) was given 0.055 cat units per kilo of body weight for 33 days without exhibiting any signs of toxicity, while dogs 5, 6, 7, and 11-E all developed severe toxic reactions while receiving intravenous doses of from 0.20 to 0.57 cat units per kilo. At autopsy, degenerative changes were seen in the heart muscles of all the dogs in the latter group. On the basis of these preliminary observations, this ratio in dogs is one to five for lanatoside C. In other words, $\frac{1}{20}$ of a cat unit of this glycoside may be given intravenously every day without apparent toxic effects over a period of 33 days. This would correspond in man to a daily injection of 5 cc.[3] (1.0 mg.) or more. Further experiments may help to establish this ratio more definitely.

COMMENT. The absence of changes in the coronary vessels of the animals reported here and by others (3–6) suggests that large doses of cardiac glycosides may produce myocardial ischemia by causing constriction of the capillaries of the muscle or by increasing the strength and duration of systole, thus interfering with the coronary flow. A less probable explanation is that these drugs cause muscle damage by direct toxic cellular injury. Since some cardiac glycosides have been found (18) to produce histological changes in the brain similar to those caused by acute anoxia, it is not unreasonable to suppose that the analogous changes in the heart which have been here described might also have been due to anoxia resulting from spasm of the coronary arteries elicited by the glycosides. It has been suggested that some of these changes in the myocardium may be caused by a loss of potassium ion. Cattell (19) has shown that the sartorius muscle of the frog quickly loses potassium ion when it is suspended in ouabain solution. This has been confirmed by Wood (20) and others (21). However, as previously pointed out, pronounced symptoms and electrocardiographic changes occur within a few minutes *after* administration of lanatoside C and often disappear within 5 to 30 minutes. The fact that these effects appear rapidly and are evanescent in character is consistent with the idea that they may be due to arterial spasm.

Essex and Visscher (2) found that *very large* doses of digitalis administered to unanaesthetized dogs reduced the coronary blood flow as measured by a thermostromuhr. Since there was no significant change in blood pressure preceding slowing of the coronary flow, it is possible that the effect is produced by spasm of the coronary arteries. The fact that there were no gross areas of infarction in the myocardiums of our dogs indicates that the effective constriction of the coronary arteries must be upon the arterioles of the coronaries. A suggested explanation of the pathological finding of atrophy of individual myofibrils is that it might be due to a low grade anoxia of long duration, but experimental proof of this assumption is lacking.

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[3] The single, complete digitalizing dose of lanatoside C in man is 1.6 mg. given intravenously. This is equivalent to a single intravenous injection of 0.266 mg. in a 25-pound dog. The digitalizing dose in man is never repeated in the same course of treatment; oral maintenance doses are given thereafter (0.25 to 1.25 mg. per day).

## CONCLUSIONS

1. The prolonged daily oral administration of folia digitalis or lanatoside C in doses from 1½ to 10 times greater than those given clinically for maintenance failed to produce morphological changes in the hearts of dogs.

2. Daily intravenous doses of lanatoside C of 0.2 mg. or more per kilo of body weight (actually 0.2, 0.25, 0.33 and 0.57 mg.) produced myocardial necrosis, fibrosis and atrophy in 44 per cent of the dogs suitable for post-mortem study.

3. Dogs given subcutaneous injections of 1.5 mg. of atropine sulfate twice daily survived daily intravenous injections of 0.5 mg. per kilo of lanatoside C almost three times as long as dogs receiving the same dosage of the cardiac glycoside but no atropine.

4. Serial electrocardiograms were taken on 15 dogs receiving daily intravenous injections of lanatoside C. All 15 dogs exhibited electrocardiographic changes, but there was no apparent correlation between pronounced electrocardiographic changes and the appearance of myocardial necrosis and fibrosis.

5. Localized spasm of the coronary vessels is suggested as a possible mechanism for the production of the myocardial necrosis and electrocardiographic changes observed in the dogs studied.

Acknowledgements. The writer is indebted to Dr. B. J. Clawson of the Department of Pathology at the University of Minnesota for help in interpreting the microscopic sections.

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# DRUG PROPHYLAXIS AGAINST LETHAL EFFECTS OF SEVERE ANOXIA

## III. *d*-, *l*- AND *dl*-PHENISOPROPYLAMINES

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In moderate doses, *dl*-phenisopropylamine (benzedrine, amphetamine) antagonizes narcotic effects of various barbiturates (1–5), urethane (5), inhalation anesthetics (6), morphine (7–9), alcohol (10–12), avertin (13, 14), chloral (15), paraldehyde (16) and other agents (3), and the catatonic effect of bulbocapnine (17). Large doses, however, are additive or synergistic with toxic amounts of barbiturates (2–4) or other depressants (18). Phenisopropylamine is ineffective in the respiratory depression of procaine intoxication (19) and synergizes the convulsant effects of camphor and cocaine (20). Its analeptic effects in avertin, chloral and pentobarbital narcoses are claimed to be only partial (18), with marked action only against the loss of the righting reflex, but the respiratory effects now appear to be well established and have been used clinically. Alles (15) has shown that *d*-phenisopropylamine has twice to four times the central stimulant action of the *l*-amine or 1.5 to two times that of the racemic form, although the optical isomers are equipotent in their peripheral actions. This finding is confirmed (21) by the relative therapeutic results with *d*- and *l*-phenisopropylamines in narcolepsy and postencephalitic Parkinson's disease.

The tolerance of guinea pigs for anoxia, measured as the persistence of respiration during exposure to low atmospheric pressures, is increased by intraperitoneal injection of 2–4 mg./kg. of *dl*-phenisopropylamine sulfate (7). Respiratory failure is also delayed in anoxic dogs given 0.025–18 mg./kg., intravenously (7). Ephedrine, norephedrine and pseudonorephedrine are reported to have similar effects in anoxic dogs (22) but epinephrine is ineffective in antagonizing lethal effects of anoxia in mice (23). It is of interest to compare the optical isomers of phenisopropylamine by a standard method (24) designed to test prophylactic effects of agents against the lethal action of severe anoxia in mice. This method involves simultaneous exposure of large groups of treated and control mice to low atmospheric pressures produced with adequate ventilation in a large decompression chamber.

Method. Mice were treated intraperitoneally with the phenisopropylamine sulfates one hour before induction of anoxia. All treated groups received 50 cc./kg. of physiologic saline as the vehicle. Injection of this amount of saline alone is without appreciable effect on resistance to anoxia (24), but control mice were treated with 50 cc./kg. of saline alone. Treated and control groups were exposed simultaneously to a reduced pressure of 523 mm. Hg, which corresponds to a simulated altitude of approximately 10,000 ft., for 10 minutes. After this plateau, the atmospheric pressure was further reduced at a rate simulating an ascent of 1000 ft./min. Exposures to anoxia were terminated when the pressure was reduced to about 141 mm. Hg, which corresponds to a simulated altitude of 40,000 ft.

Results. Lethal effects of anoxia in the various groups of mice are expressed as mortality ratios (no. succumbing/no. used) in table 1. The first dose tested was 10 mg./kg. of the three phenisopropylamine sulfates, which is well below their intraperitoneal $LD_{50}$ in mice (15) and but 55% of the highest intravenous dose of *dl*-phenisopropylamine stated to be beneficial in anoxic dogs (7). However, nearly all of the treated mice died at a level of anoxia killing only 11 of 50 untreated mice simultaneously exposed. Thereafter, in experiments with lower doses, no attempt was made to obtain 50% mortality of the control groups, which is otherwise a desirable endpoint for the standard method (24). Mortality was uniformly greater in all groups of treated mice than in their appropriate controls.

Mice treated with 10 mg./kg. of the phenisopropylamine sulfates showed intense spontaneous motor activity, grossly equal in the three groups treated with different isomers. This excessive activity lasted for the entire period before and during exposure to anoxia. A very few of the mice appeared to be exhausted before exposure to anoxia, but none was moribund. With 2 mg./kg., there was also marked hyperactivity in all treated groups, but only a minor increase in voluntary activity was noted in groups treated with 0.2 mg./kg.

TABLE 1

*Influence of the phenisopropylamines on lethal effects of anoxia, in mice*

| DOSE OF AMPHETAMINE SULFATES | CONC. OF INJECTED SOLUTIONS | MORTALITY RATIOS | | | |
|---|---|---|---|---|---|
| | | *l*- | *d*- | *dl*- | Controls |
| *mg./kg.* | *per cent* | | | | |
| 10.0 | 0.02 | *47/50* | *50/50* | *50/50* | 11/50 |
| 2.0 | 0.004 | 6/30 | *16/30* | *12/30* | 5/30 |
| 0.2 | 0.0004 | 15/30 | 11/30 | *18/30* | 9/30 |

Italicized mortality ratios represent values significantly different in the statistical sense from mortality ratios of the control groups, with a $p$ of 0.05 or less.

Discussion. Under the conditions employed, none of the phenisopropylamines has any prophylactic effect against lethal actions of anoxia in mice when administered in doses equivalent to therapeutic doses in man and in doses ten or fifty times larger than these. Superficially, the present results directly contradict those reported for anoxic guinea pigs and dogs (7). The cause of this difference is probably related to technical differences rather than to species variation. The various physiologic processes involved in lethal actions of acute progressive anoxia presumably have a markedly different relative importance in the fulminating anoxia to which guinea pigs and dogs were exposed. Certain other substances which normally act as efficient respiratory stimulants have also been found to be ineffective as prophylactics in acute anoxia and the various factors concerned in prophylaxis against anoxia have been discussed (23).

To test the actuality of the discrepancy between results in fulminating and acute anoxia, experiments similar to those of Binet (7) were repeated in cats.

Two cats showed marked respiratory stimulation after intravenous injection of 2 mg./kg. of the phenisopropylamine sulfates given when nearly complete respiratory failure was attained after a period of inhalation of nitrogen through soda-lime. Two dogs also showed marked increases of rate and amplitude of respiration following intravenous injection of the same dose of the isomers after they had been rendered cyanotic with large doses of morphine and pentobarbital. These latter results confirm the report of Handley and Abreu (9). Tachyphylaxis was noted and the order of repeated injections of the isomers was varied accordingly. About 30 minutes were allowed between injections of the different isomers, but both the *d*- and *l*-isomers ultimately failed to stimulate respiration after repeated injections. No large difference between respiratory effects of the two isomers could be recognized, and if a difference exists, its quantitative measurement would require observation in a large series of animals.

In the instances specially noted in table 1, the phenisopropylamines exhibited an unquestionably harmful effect in anoxia, with statistically significant differences in mortality ratios (25). In all other instances, the mortality ratios in treated groups of mice were higher than those of the appropriate control groups, although not significantly so. This harmful effect is most probably due to the increased oxygen consumption occasioned by hyperactivity, since it is most marked with doses greatly affecting motor activity. In rats, subcutaneous injection of 3 mg./kg. of *dl*-phenisopropylamine results in a very large increase in spontaneous activity during the first hour, decreasing to normal in about four hours (26). Basal metabolism is not markedly stimulated in man after full therapeutic doses of *dl*-phenisopropylamine (27) and moderate activity of mice during exposure to anoxia is not harmful (24), but intense hyperactivity favors the occurrence of terminal convulsions in anoxic mice (23). That factors other than simple increase of oxygen consumption may play an important part is indicated by the results with mice treated with 0.2 mg./kg. of the phenisopropylamines.

The relative effects of the different isomers at the two higher dosage levels suggest that the *d*-isomer is more detrimental in anoxic mice than the *l*-isomer. Alles (15) states that differences in motor stimulation in mice are not relatable to differences in optical isomerism and no gross differences were noted in the degree of hyperactivity of mice treated with equivalent doses of the different isomers in the present study, but among 75 amines studied with rats (28) *d*-phenisopropylamine had the greatest effect on spontaneous activity, followed by the racemic and *l*-isomers. *d*-Phenisopropylamine is a more effective analeptic than the *l*-isomer in paraldehyde narcosis of mice (16) and in chloralized rabbits (15). As a class, cortical stimulants (23) are not uniformly active as antagonists to lethal effects of anoxia in mice, and other physiologic effects of these agents may be of more significance. Alles (15) has shown that the peripheral effects of the three phenisopropylamines are similar.

In order to determine if peripheral effects of the isomers are also similar under the various abnormal conditions of the autonomic nervous system which might be encountered in anoxia, the comparison presented in figure 1 was made. Ef-

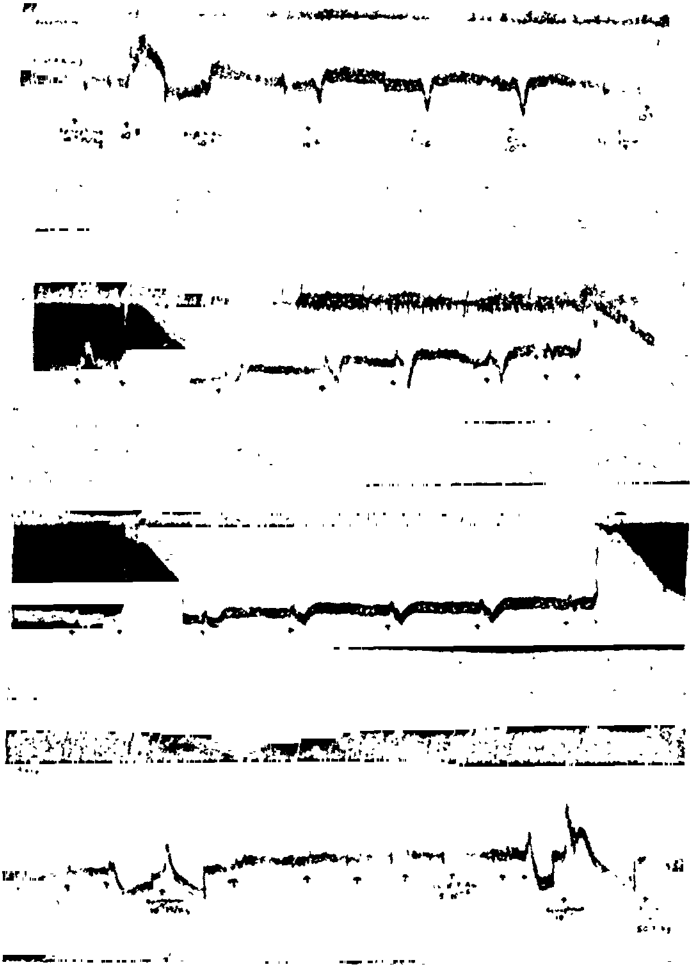

Fig. 1. Effects of Isomers of Phenisopropylamine on Carotid Pressure in the Dog

Female dog, 20.5 kg., pentobarbital narcosis.

Top record: intravenous injections of epinephrine, $10^{-9}$ mols/kg.; $10^{-8}$ mols/kg.; *d*-phenisopropylamine, $10^{-6}$ mols/kg.; *dl*-, $10^{-6}$ mols/kg.; *l*-, $10^{-6}$ mols/kg.; *d*-, $10^{-5}$ mols/kg.; epinephrine, $10^{-9}$ mols/kg.; and $10^{-8}$ mols/kg. Then, not shown, 7.5 mg./kg. of atropine sulfate intravenously in 5 divided doses, each of the doses followed by $10^{-8}$ mols/kg. of epinephrine. Cardiac irregularity due to epinephrine became progressively less but did not disappear. A 2-hour interval was allowed before the second record. Acetylcholine then produced a slight pressor effect.

Second record: injections as noted in top record. Then, not shown, 10 mg./kg. of cocaine hydrochloride, subcutaneously. 15 minute interval before the third record.

Third record: injections as noted in top record. Then, not shown, 10 mg./kg. of piperidinomethylbenzodioxane, 933F, intravenously. Slight depressor response to 0.05 mg./kg. of acetylcholine iodide. 10 minute interval before the fourth record.

Fourth record: injections as noted in top record except for interposed injections of epinephrine $10^{-7}$ mols/kg. and *dl*-phenisopropylamine 5 x $10^{-6}$ mols/kg., as indicated on the record, and final injections of epinephrine $10^{-7}$ mols/kg. and acetylcholine iodide 0.05 mg./kg., as indicated on the record.

Interval between individual injections throughout, 10-20 minutes. Time marker on fourth record indicates 15-second intervals; synchronous kymograph maintained at constant speed for all records.

fects of the isomers and of epinephrine were noted first in a pentobarbitalized dog, confirming Alles' findings (15). The observations were repeated in the same animal after administration of atropine, cocaine, and a sympathicolytic agent, 933F, respectively. In each case, effects of the different phenisopropylamines on circulation were equivalent. An appreciable tachyphylaxis occurred despite a long interval between injections of the isomers and was controlled by repetition of injection of the first isomer given, after the others had been administered. A diminution of the pressor effect of epinephrine after injection of the phenisopropylamines was also apparent. It may be concluded that the circulatory effects of the three isomers are similar under widely different conditions of the autonomic nervous system.

Although the results with anoxic mice indicate that the phenisopropylamines are harmful even at dosage levels corresponding to therapeutic amounts used in man, no clinical implications from these findings are warranted. Conditions present in the moderate anoxia occurring in many diseases of man are not comparable to those of the lethal levels of anoxia used in the present study, and effects of drugs on mice cannot be employed alone as a basis for conclusions concerning the clinical usefulness of drugs. It is felt that phenisopropylamine deserves further trial as an analeptic agent in man, particularly in intoxications such as morphine poisoning (9), but that its use in severe anoxia and in aviation practice should be approached more cautiously than would be suggested by a report (7) of its effects in fulminating anoxia.

## SUMMARY

*dl*-Phenisopropylamine (benzedrine) and its optically active isomers have no prophylactic value against lethal actions of acute anoxia in mice, when administered in doses comparable to those used therapeutically in man, and are significantly detrimental to anoxic mice when administered in higher sublethal amounts.

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shows no evidence of necrosis, with the exception of a few scattered areas of slight damage. After four doses the kidneys likewise suffer very little necrosis.

If the rats which have received four or five doses of 35 mgm./kgm. of gold sodium thiosulphate are then given 100 mgm./kgm., an amount to which they are now tolerant, their kidneys, when examined 24 hours later, show evidence only of mild cytoplasmic damage. (Fig. 1.) The majority of the nuclei are intact. The picture is similar to that of a rat receiving four doses of 35 mgm./kgm. without the additional dose of 100 mgm./kgm. However if one examines the kidneys of a normal rat 24 hours after injecting a single dose of 100 mgm./kgm. of gold sodium thiosulphate one sees a really severe nephrosis. (Fig. 2.) The picture is similar to that of the kidney of rats following a single dose of 35 mgm./kgm., only more severe, the necrosis being sufficient to result in the death of the animal within three days.

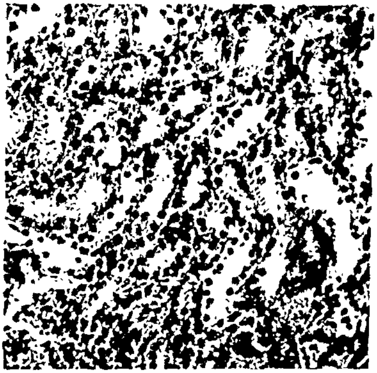

FIG. 1

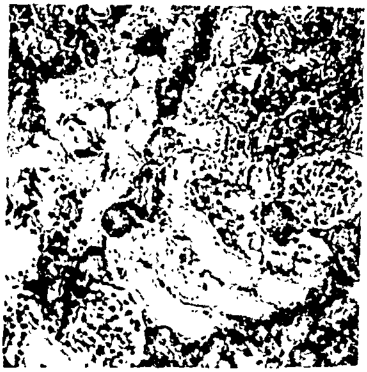

FIG. 2

FIG. 1. PHOTOMICROGRAPH OF THE KIDNEY OF A RAT WHICH HAD RECEIVED 35 MGM./KGM. OF GOLD SODIUM THIOSULPHATE INTRAMUSCULARLY EVERY OTHER DAY FOR 5 DOSES AND THEN RECEIVED 100 MGM./KGM. OF GOLD SODIUM THIOSULPHATE TWO DAYS AFTER THE LAST CHRONIC DOSE.

The rat was killed 24 hours after the 100 mgm./kgm. dose. Magnification × 160.

FIG. 2. PHOTOMICROGRAPH OF THE KIDNEY OF A CONTROL RAT 24 HOURS AFTER RECEIVING A SINGLE DOSE OF 100 MGM./KGM. OF GOLD SODIUM THIOSULPHATE INTRAMUSCULARLY. MAGNIFICATION × 136

In the group of rats receiving 20 mgm./kgm. doses every other day, the sequence of events is similar to that described above. The necrosis caused by the first injection of 20 mgm./kgm. is not quite as severe as that caused by a single injection of 35 mgm./kgm., but the subsequent repair is similar, and the kidney of a rat receiving repeated doses of 20 mgm./kgm. of gold sodium thiosulphate intramuscularly followed by 100 mgm./kgm. of gold sodium thiosulphate shows either a very slight degree of damage or none at all.

Repeated injections of 10 mgm./kgm. do not permit survival following 100 mgm./kgm. of gold sodium thiosulphate, but they enable the animals to live longer than normal rats injected with 100 mgm./kgm., indicating some tolerance. Histological examinations of their kidneys reveal some interesting findings. The first dose of 10 mgm./kgm. results only in a very slight necrosis in some regions of the convoluted tubules, but animals which have received repeated doses of 10 mgm./kgm. every other day show kidneys which are essentially normal. If rats given repeated doses of 10 mgm./kgm. are then given 100 mgm./kgm. of gold sodium thiosulphate and their kidneys examined 48 hours later, one finds a very severe necrosis in the region of Henle's loops, and many casts in the collecting tubules, but the cortical areas of the kidney show no necrosis. These results indicate that these small doses of gold sodium thiosulphate, although they caused only slight damage to the epithelial cells in the region of the renal cortex, nevertheless must have had some effect on them, because later the large dose of 100 mgm./kgm. left these cells undamaged, indicating their increased resistance to gold. The necrosis of the remainder of the kidney was sufficient to cause the ultimate death of the animal.

If rats which were made tolerant to gold sodium thiosulphate by repeated injections of 35 mgm./kgm. were given very large doses, such as 200 mgm./kgm., these rats died within 5–7 hours, with relatively intact kidneys. Normal rats receiving similar doses also died within 5–7 hours, but with severe renal necrosis. The cause of death with these large doses is apparently not related to renal damage, but is probably due to circulatory collapse as a result of capillary or cardiac damage, or central paralysis (4).

In the course of these investigations to study the mechanism of tolerance formation to gold sodium thiosulphate it is worthwhile mentioning that the rôle of the liver was excluded as a factor by subjecting tolerant rats to partial hepatectomy or carbon tetrachloride poisoning before testing them for tolerance. Neither partial hepatectomy nor functional liver damage was found to interfere with the tolerance to gold sodium thiosulphate. In none of the rats receiving either small or large doses of gold sodium thiosulphate was any liver damage evident.

Discussion. These results show that rats receiving a single dose of 20 to 35 mgm./kgm. of gold sodium thiosulphate intramuscularly suffer a degree of damage to the renal tubular epithelium which can be repaired. In some animals, especially those receiving 35 mgm./kgm., the damage is sometimes more severe and may cause the death of the rat. Animals which survive the first dose of gold sodium thiosulphate are resistant to subsequent injections, and these injections have only the slightest effect on the repaired epithelium of the kidney tubules. In fact the epithelium becomes so resistant to the damaging effect of the gold salt that 100 mgm./kgm. can be tolerated with no significant effect on the histology of the kidney although this dosage in a normal rat results in a widespread, fatal nephrosis.

Tolerance formation as a result of specific tissue resistance has been described by other authors. MacNider (5) showed that dogs that survived toxic doses of uranium nitrate were resistant to lethal doses of bichloride of mercury, presumably due to a metaplastic repair of the uranium-damaged epithelium of the proximal convoluted tubules of the kidney resulting in a type of cell resistant mediate between those of the *3,5*-dinitrophenylurethanes of cannabinol and synthetic tetrahydrocannabinol. Hydrolysis of this derivative gave a material which Walton (10) found to be two to three times as active as synthetic tetrahydrocannabinol in dogs, but only one-fifth to one-third as active as a potent red oil fraction.

More recently Wollner, Matchett and coworkers (11), working with an acetylated oil distillate of charas of Indian origin, were able to separate out four high-vacuum distillation fractions of constant refractive index and optical rotation. Determination of the potency of these acetate fractions on dogs by Loewe (11) showed them to be ten to fifteen times as active as synthetic tetrahydrocannabinol. Acetyl and C,H analyses agreed well with calculated values for an acetyltetrahydrocannabinol. Hydrolysis with acid or ammonia resulted in a product with lower optical rotation, and the physiological activity was but six to ten times that of synthetic tetrahydrocannabinol when tested on dogs by Loewe (11), which represents an activity about that of a potent red oil.

Valuations of the physiological activity of crude extracts or other preparations of Cannabis have been chiefly made by the Dixon test (12) of measuring the minimum amount of drug necessary to produce motor incoordination in a dog. Some workers have used the Gayer test (13) of measuring the minimum amount of drug necessary to produce corneal anesthesia in a rabbit. To obtain results which have any quantitative value by either method, the testing must have been done on the basis of determining the dosage of the unknown preparation which produces the same minimal effect as a given dose of a standard preparation in that particular animal. This technic has been well described as bioassay by the method of approximation by Loewe (14, 15), but the expression of percentage error used is more an expression of variation in test technic than an expression of biological variation.

In connection with our own efforts to isolate physiologically active fractions from Cannabis preparations (9), the Gayer rabbit test was initially set up as the bioassay method, because Walton, Martin and Keller (16) had found a close correspondence between the Dixon dog test and the Gayer rabbit test in working with distillation fractions of Cannabis. The cumbersomeness, statistical inadequacy, and tediousness of the dog test also affected our choice. However, after a number of experiences in which relatively mild handling procedures appeared to result in a practical disappearance of corneal anesthetic activity in rabbits, certain work was repeated using dog test methods, with contradictory results. It became apparent that the two methods of testing were measures of different types of activity, and since the dog test activity was the more stable, our attention from the standpoint of isolation was then limited to fractions exhibiting such activity.

The different ratio of dog and rabbit activity of various hemp extracts is considerable, but might be simply explainable on the basis of there being different distributions of various cannabinol derivatives in such extracts, and that these derivatives vary somewhat in their relative potencies when tested in the dog and the rabbit. In the present work, we were able to carry out some inactivation experiments with extracts of charas that are so definite in the change of relative dog and rabbit activities as to require the conclusion that another active principle is probably present. This principle would be characteristically very active in producing corneal anesthesia in rabbits as demonstrated by the Gayer test.

Bioassays on Crude Extracts. During 1939 two batches of alcoholic extracts of Cannabis of American origin were generously supplied for our studies by the Narcotics Laboratory of the Treasury Department through the cooperation of H. J. Anslinger, H. J. Wollner and John R. Matchett. These extracts were made from Minnesota wild hemp grown during the 1938 and 1939 season, respectively. Both batches were prepared in similar manner from air-dried material, after separation of coarse stems, by extraction with ethanol until a concentration of about 2 g. per 100 cc. was reached, then this extract evaporated below 50°C. to give a final product containing 23-27 g. total solids per 100 cc., with each cc. equivalent to about 4 g. hemp.

The 1938 hemp extract was received in March 1939, assayed in June 1939, and again in June 1940, after standing at room temperature following some preliminary testing. Eight dogs were initially selected for the assay work, and six of these were available for the second tests. White rabbits weighing 1.5-2 kg. were used, and *a different group of animals was used for the second tests. The testing* was done with a standard solution made up to 100 mg. total solids per cc., and the material administered orally after dilution with water.

*1938 Minnesota Hemp*

| DOGS, ORAL | MG. PER KG DOSE | | | RABBITS, ORAL | MG. PER KG. DOSE | | |
|---|---|---|---|---|---|---|---|
| | 20 | 40 | 80 | | 10 | 20 | 40 |
| 6/39 | 0/8 | 5/8 | 8/8 | 6/39 | 1/9 | 5/9 | 9/9 |
| 5/40 | 2/6 | 5/6 | | 6/40 | | 0/4 | 0/4 |

The 1939 hemp extract was received the latter part of October, very shortly after its preparation. Chemical studies had indicated that addition of benzene to the initial ethanol extract, then partial evaporation, with repetition of this process several times, yielded an anhydrous benzene extract that was more suitable for further chemical work. This preparation was of sufficient purity so that its solids gave a solution in acetone which could be injected intravenously. Comparison of the differences between the intravenous and oral routes of administration, using benzene dehydrate extracts of 1938 Minnesota hemp, had indicated intravenous tests to be about ten times as sensitive for rabbits.

*1939 Minnesota Hemp*

| DOGS, ORAL | MG. PER KG. | | RABBITS, ORAL | MG. PER KG. | | RABBITS, IV | MG. PER KG. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 40 | | 20 | 40 | | 2 | 4 | 8 | 16 |
| 1/40 | 3/8 | 6/8 | 1/40 | 0/4 | 0/4 | 1/40 | 0/4 | 0/4 | 0/4 | 0/4 |

In December 1940, another batch of Cannabis extract was received from the Narcotics Laboratory that was obtained from hemp grown in Illinois during 1935, 1936 and 1937, then stored protected from weather until October 1940, when it was extracted. Methanol was used for making this extract, instead of ethanol (as had been used previously), and the methanolic extract was concentrated at atmospheric pressure until it contained about 67 g. total solids per 100 cc., which exposed it to considerably higher temperatures than earlier extracts. The bioassays were again run on benzene dehydrate extracts of this material so that intravenous tests could also be carried out upon rabbits.

*1935–1937 Illinois Hemp*

| DOGS, ORAL | MG. PER KG. | | RABBITS, ORAL | MG. PER KG. | | RABBITS, IV. | MG. PER KG. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 40 | | 20 | 40 | | 2 | 4 | 8 | 16 |
| 1/41 | 2/6 | 5/6 | 1/41 | 0/4 | 0/4 | 1/41 | 0/4 | 0/4 | 0/4 | 0/4 |

The dog and rabbit tests of the 1938 and 1939 Minnesota hemp extracts show that storage of these extracts can cause a greater deterioration of rabbit activity than of dog activity. Further, due to yearly variation, or some minor details in making extracts (such as the benzene dehydration process), rabbit activity may be absent from certain extracts, at least within practicable test limits. The tests with the Illinois hemp extracts introduced a geographical variable along with age of the plant material, in addition to some variables in details of making extracts, but it is clear that, no matter what the cause, there is a real difference between the relative dog and rabbit activity of such extract in comparison with the 1938 Minnesota hemp extract.

HEAT INACTIVATION OF CHARAS EXTRACTS. As it was not possible for us to obtain any further American hemp extracts that exhibited any considerable degree of rabbit activity, several series of experiments were carried out with ethanol extracts of some charas of Indian origin. This material was kindly supplied by the Narcotics Laboratory of the Treasury Department in July 1940. This type of material, as has been noted by others, is most satisfactory for the preparation of potent red oil distillates from ethanol extracts which exhibit both dog and rabbit activities.

In considering the various factors that might account for loss of rabbit activity on storage of the 1938 Minnesota hemp extract, or the inactivity of the later studied extracts, we decided that time and temperature in the presence of air might be the controlling factors. Initial experiments were tried, which developed the following procedure for demonstrating the loss of rabbit activity on heating ethanol extracts of charas in the presence of air:

Two grams charas were ground with some 95% ethanol, the extract filtered and extraction repeated until 200 cc. of filtered extract were obtained. After evaporation under reduced pressure, 100 cc. of this extract gave a residue of 0.334 g. solids, and these were taken up to 3.34 cc. with acetone, and tested. The remaining 100 cc. extract was placed in a flask under a reflux condenser, the flask placed in a water bath at 50°C., and a gentle current of air bubbled through the solution for four hours. After evaporation under reduced pressure the residual solids were taken up to 3.34 cc. with acetone, and tested.

| RABBITS, INTRAVENOUS | MG. PER KG DOSAGE | | | |
|---|---|---|---|---|
| | 2 | 4 | 8 | 16 |
| Unheated extract | 0/4 | 6/6 | 6/6 | 4/4 |
| Heated extract | 0/4 | 0/6 | 0/6 | 0/4 |

Having developed a satisfactory technic for the heat inactivation of charas extracts, the experiment was repeated on a larger scale, and at double the former concentration of the extract, to obtain sufficient material to carry out both dog and rabbit tests. The dog tests were carried out by administering the dosage made up in ethanol via a stomach tube with water, and comparisons were noted on an individual basis, rather than on the animals as a group.

Thirty grams charas were ground with some 95% ethanol, the extract filtered and extraction repeated until 1500 cc. of filtered extract were obtained. Two portions of 375 cc. each were evaporated under reduced pressure, and the residues each weighed 2.62 g. One was taken up to 26.2 cc. with ethanol (Ia), the other to 26 2 cc. with acetone (Ib), then tested. The remaining 750 cc. of extract was placed in a flask in a water bath at 50° C. and air bubbled through for four hours. This solution was then divided into two parts, each evaporated, and the residues taken up, one to 26.2 cc. with ethanol (IIa), and the other to 26.2 cc. with acetone (IIb), then tested.

| DOGS, STOMACH TUBE | Ia—MG. PER KG. | | | IIa—MG. PER KG. | | |
|---|---|---|---|---|---|---|
| | 4 | 8 | 16 | 4 | 8 | 16 |
| Black pup | 0 | 0 | x | 0 | x | x |
| White female | 0 | 0 | x | | 0 | x |
| Brown and black | 0 | 0 | x | | 0 | x |
| Airedale | 0 | 0 | x | | 0 | x |

0 denotes no reaction; x denotes incoordination reaction.

| RABBITS, INTRAVENOUS | Ib—MG. PER KG | | | | IIb—MG. PER KG. | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 8 | 16 | 2 | 4 | 8 | 16 |
| 6/41 | 0/4 | 3/3 | 4/4 | | | 0/4 | 0/4 | 0/4 |

Discussion. As a result of the heating of ethanol extracts in the presence of air, under the conditions used, it is obvious that there is a marked deterioration of the extracts with respect to rabbit activity (to one-eighth or less), without any corresponding change in the dog activity. The mechanism of the production of the test activities in the dog or in the rabbit is not known, but it seems not unlikely that two different physiological mechanisms are involved. The simplest explanation for the change on heating would then appear to be that some active principle or principles that exhibit the rabbit corneal anesthesia activity in marked degree are present and are deteriorated much more rapidly than are the active principles that exhibit the dog incoordination activity.

The conclusion that this rabbit activity principle is different from the principles that have already been found in Cannabis is not altogether certain because, unfortunately, the principles that have been isolated in a probably pure form have not been quantitatively evaluated upon rabbits. It is likely that another principle is involved, however, since all active principles that have been isolated are strongly active in producing incoördination in dogs, and a considerable destruction of any of these isolated principles would have been shown by tests made on dogs. Of interest in this connection is the finding of Loewe and Modell (8) that tetrahydrocannabinols produced by chemical isomerization of isolated cannabidiol are relatively less active in rabbits than in dogs when valued against a standard red oil. They drew the conclusion that Cannabis extracts must therefore contain a principle other than these tetrahydrocannabinols.

A possible alternative hypothesis that could explain the presently reported observations, but which seems far less likely on *a priori* grounds, could be advanced on the basis that no new active principle is involved, but that both oxidized and unoxidized forms act almost equally in the dog, while only the unoxidized form acts in the rabbit.

## SUMMARY

1. Alcoholic extracts of Cannabis of American origin may vary considerably in their relative activities, as measured by the dog incoordination and rabbit corneal anesthesia tests.
2. This variation may be due to variation in the extracted materials, but can be entirely due to exposure to air under moderate conditions of time and temperature.
3. Deterioration of Cannabis extracts under such conditions may lead to loss of significant activity in the rabbit without notable loss of activity in the dog.
4. The corneal anesthesia activity in the rabbit of Cannabis extracts would therefore appear to be due to an active principle not yet isolated, for the active substances that have been isolated all exhibit considerable dog incoordination activity.

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# NITRITES

## VIII. BLOOD-NITRITE CONTENT OF MAN AND OTHER SPECIES[1]

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A search into the literature has revealed that very little, if anything, is known concerning the normal physiological significance of the nitrite ion, i.e., whether or not it exists as such in the circulation and if it does, what rôle it plays and what factors influence its concentration in the blood. Studies by several investigators indicate that the nitrite ion does exist normally in the blood. Stieglitz and Palmer (1, 2) detected it in the blood of normal human subjects but obtained consistently negative results when dog and rabbit blood was analyzed. These observers flequently noted the absence of nitrite in human blood during the warm months of the year. They reported an average concentration of $1:10^8$ for 26 subjects during the cool months. An error in the mathematical treatment of their data was discovered and Stieglitz has agreed that their values require correction (3). The corrected values indicate a blood-nitrite concentration in the order of magnitude of $1:10^7$.

METHODS *Quantitative determination of blood nitrite.* Stieglitz and Palmer (1) developed a colorimetric test for blood nitrite, employing $\alpha$-naphthylamine and the disodium salt of $\beta$-naphthylamine-6,8-disulfonic acid ("amino G" acid), which they claimed to be more sensitive and more accurate than the Ilosvay reaction. The method of Stieglitz and Palmer, as studied in this laboratory, was found no more sensitive than the Ilosvay reaction for nitrite and is far less satisfactory.

*Preparation of protein-free blood filtrate.* As nitrites are unstable in acid media, the method of removing the blood proteins must avoid an acid solution. Somogyi (4) devised a satisfactory means for accomplishing this by using zinc sulfate and sodium hydroxide. The high dilution of blood resulting after the addition of the reagents in the concentrations prescribed by him is undesirable here, however, since the nitrite ion is present in extremely minute amounts in normal blood. A modification of the method of Stieglitz and Palmer was therefore employed.

By this method, 1 volume of blood is thoroughly mixed with 2.5 volumes of 4.5% zinc sulfate solution in an Erlenmeyer flask One-half volume of 1 N sodium hydroxide is added and the mixture again shaken. Centrifugation produces a clear filtrate. If immediately after blood is withdrawn from a blood vessel it is placed in the flask containing the zinc sulfate, no special anticoagulant need be employed.

For most of the analyses on normal blood, 10 cc. were studied which yielded 20 cc. of filtrate (equivalent to 5 cc. of whole blood).

*Production of color reaction.* A definite quantity of the clear, protein-free filtrate is placed in a tall 50 cc. Nessler tube containing 2 cc. of each diazotization reagent, α-naphthylamine[3] and sulfanilic acid.[4] The Nessler tube is then placed in water maintained at 75–85° C. and allowed to remain for 10 minutes. The tubes containing the standards for comparison are prepared in exactly the same manner, i.e., a quantity of filtrate exactly the same as that obtained from blood is employed, such filtrate being obtained by mixing an equal amount of standard solution, in place of blood, with zinc sulfate and sodium hydroxide.

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[1] The expense of this investigation has been defrayed in part by a grant from the Bressler Research Alumni Fund of the University of Maryland.

[2] The material contained in this paper is part of a thesis submitted by Maurice M. Rath to the faculty of the Graduate School of the University of Maryland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

*Preparation of standards for comparison.* Merck's reagent grade granular sodium nitrite (99.4%) was employed. With due allowance for the slight amount of impurity, a stock solution was prepared containing 1.0 milligram of the nitrite ion per 1.509 cc. of solution by dissolving 1.0 gram of sodium nitrite (99.4%) in 1000 cc. of nitrite-free water. From this stock solution prepared weekly, successive dilutions were made daily to obtain the necessary standards for comparison.

*Colorimetric reading.* Tall 50 cc. Nessler tubes and a Nesslerimeter[5] were used for this purpose and provided a method which is rapid, convenient and accurate. Chances of contamination by this procedure were minimized because the solution containing the nitrite ion can be mixed with the azo dye reagents in a Nessler tube, the mixture then heated in a water bath, and the color so developed compared with standard solutions without transferring to any other container. As many standards of varying concentrations can be employed as are necessary and placed in the Nesslerimeter for matching colors.

Colorimetric matching, by means of the Nesslerimeter, was made on samples of normal blood. When the concentration of nitrite is more than 15 gamma per 100 cc. the ordinary colorimeter with 50 millimeter cups is suitable. In the latter case, fewer standards of varying concentration need be employed.

By the method herein described, readings were reproducible when analyses were made on 10 samples of blood withdrawn from a single pool of blood. During all analytical procedures scrupulous caution must be exercised to avoid contamination of the sample with extraneous nitrite from air, water and glassware.

DETERMINATION OF NITRITE IN THE BLOOD OF THE DOG, STEER, MONKEY, AND MAN. By the method described in the foregoing pages for determining minute quantities of nitrite, the blood of various species of animals was analyzed. The results of this investigation for the dog, cow, Rhesus monkey, and man, respectively were as follows:

For 69 dogs, the average nitrite level in the blood obtained either from the external jugular vein or femoral artery was 9.5 gamma, plus or minus a standard error of 0.39, per 100 cc. of blood. The high value was 18 gamma and the low 4 gamma. Attempts to alter the blood-nitrite level by several methods in dogs met with failure. These methods included prolonged fasting and feeding amino acids, ammonium chloride, potassium nitrate and certain organic nitrates. Sodium nitrite alone readily raised the blood-nitrite level.

The blood of the steer, collected from the jugular vein at the time of slaughter, contained an average 9.4 gamma of nitrite per 100 cc. in 5 animals with a standard error of 1.25 gamma. The high value was 13 gamma and the low 7 gamma.

Fourteen monkeys from which samples of blood were obtained from the ventricles of the heart had a nitrite concentration averaging 11 gamma per 100 cc. plus or minus a standard error of 0.5. The high value was 15 gamma and the low 8 gamma.

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[3] α-naphthylamine solution is prepared by placing 100 milligrams of the base (Eastman Kodak Co., N. Y.) in boiling 5 N acetic acid. Store in tightly stoppered glass container.

[4] Sulfanilic acid solution is prepared by dissolving 0.5 gram in 150 cc. of boiling 5 N acetic acid.

[5] The Nesslerimeter (Fisher Scientific Company, Pittsburgh, Pa.)

A study of a series of 170 human subjects revealed an average of 9.45 gamma of nitrite plus or minus a standard error of 0.47 per 100 cc. of blood withdrawn from the antecubital vein. The subjects were patients hospitalized for traumatic injuries, operative treatment etc. Both sexes, white and colored races, all age levels and various blood pressure levels were represented in this series of individuals. These data are summarized in table 1.

On the basis of these results it appears that nitrite is present normally in the blood of the dog, steer, monkey, and man, and it exists in approximately the same detectable concentration in all these species, namely 10 gamma per 100 cc. of blood, or one part in ten million.

TABLE 1

*Blood nitrite concentration in man—summary*

Total number of subjects, 170

| | | HIGH | LOW |
|---|---|---|---|
| Mean nitrite in gamma per 100 cc. blood | 9.45 | 29 | 0.5 |
| Standard deviation | 6.25 | | |
| Standard error | 0.48 | | |
| Mean systolic blood pressure in mm. Hg | 152 7 | 240 | 80 |
| Standard deviation | 32.4 | | |
| Standard error | 2.4 | | |
| Mean diastolic blood pressure in mm. Hg | 92.8 | 180 | 20 |
| Standard deviation | 21.9 | | |
| Standard error | 1.7 | | |
| Mean pulse pressure in mm. Hg | 62.6 | 120 | 20 |
| Standard deviation | 20.0 | | |
| Standard error | 1.5 | | |
| Mean age in years | 51 4 | 91 | 9 |
| Standard deviation | 18.7 | | |

Supporting the findings of Krantz, Carr and coworkers (5) it was found that the blood of the dog contains nitrite. These investigators did not determine the exact quantity of nitrite in blood in various species. They were interested in learning the relative increase in nitrite concentration after the administration of drugs to dogs. Stieglitz and Palmer (2) were unable to detect nitrite in the blood of the dog. Also, these investigators reported that human blood frequently contains no nitrite in warm weather. It is to be noted that 90% of the series of human subjects represented in table 1 was obtained during the summer months in Baltimore. Whether this detectable quantity represents all of nitrite present in blood is as yet unknown.

THE RELATIONSHIP OF ARTERIAL TENSION AND AGE TO BLOOD-NITRITE LEVEL. A statistical analysis of the data obtained on the 170 people set forth in table 1 indicates that the correlation between arterial tension and blood-nitrite level is not significant. The chi square test applied to the age-nitrite data for 170 subjects reveals that significance may be attached to the difference in blood nitrite concentration of younger (54 years of age or below) and older subjects (55 years of age and over). This test gives a $P$ between 0.02 and 0.01 which means that in only 2 (or less) chances out of 100 can the difference observed occur by chance. When the subjects are segregated into young and old and their mean blood-nitrite levels compared, the difference also is statistically significant. The difference of 4.23 gamma per 100 cc. between young and old is 4.1 times the standard error of the difference. The odds against the occurrence of a deviation as much as 4 standard errors is great.

An additional series of 32 aged subjects was studied with respect to nitrite concentration of the blood. The average age for this new group was 73.5 years with a mean nitrite concentration of 5.84 gamma per 100 cc. of blood (standard deviation 2.0 and standard error 0.38). With 1 or 2 exceptions, these subjects had blood pressures within normal limits. Here is further indication that low nitrite levels are not necessarily associated with high arterial tension. The explanation for the lower average nitrite level in older individuals is not yet forthcoming.

Evidence that the oral administration of large amounts of nitrate is followed by appreciable fall in blood pressure is lacking. But this does not preclude the possibility that nitrite is formed in minute quantities during some physiological process involving the tissue nitrate (Whelan (6) has reported an average of 1 mgm. of nitrate nitrogen is present per gram of tissue), that it enters the circulation wherein it is maintained at a fairly constant concentration. A survey of the literature has revealed a number of findings from various branches of biology and chemistry which bear on this problem and which may not have been heretofore collected and correlated.

Various types of foods contain nitrates. Some of the normal intestinal flora are capable of reducing nitrate to nitrite, especially *B. coli* and *lactis-aerogenes* (Zobell (7) Stieglitz and Palmer (8) Aubel and coworkers (9 and 10). Some bacteria oxidize ammonia to nitrite (Zinsser and Bayne-Jones (11) and various intestinal strains destroy nitrite by converting it to nitrate (Zobell (7) and Stieglitz and Palmer (8)).

A number of investigators have found that plant enzymes convert nitrate to nitrite. They believe that plants containing nitrates yield nitrites by action of both plant and animal enzymes (Kastle and Elvove (12) Bernheim and Dixon (13).

Animal tissues also apparently contain systems capable of reducing nitrates to nitrites. Stephanow (14) showed that tissues which normally contained no nitrite were able to form it when ground with nitrates.

Bernheim and Dixon reported that the livers of the sheep, pig, rabbit, dog, chicken, rat and guinea pig reduce nitrates very readily and that the liver appears to be the main site of this phenomenon. They demonstrated by *in vitro* experiments that nitrate is reduced during the process of oxidation of aldehyde, xanthine, adenine by oxidases of liver tissue. The amount of nitrite produced per time unit in the presence of different substances was exactly parallel to the rate of reduction by methylene blue. In other words the nitrate readily takes the place of methylene blue as a hydrogen acceptor in other oxidation-reduction systems (Dixon and Thurlow (15), Bach (16), Openheimer and Stern (17)). It is interesting to note that Kastle and Elvove (12) found that alcohols and aldehydes also greatly accelerate the reduction of sodium nitrate by plant extracts. At that time they suspected that nitrates play a vital rôle in biochemical oxidations and reductions.

Aubel believes that nitrates are important in the mechanism of biological oxidations and that under certain processes of dehydrogenation in cellular metabolism, they, along with oxygen, constitute the only acceptors of hydrogen. Baudisch (18) discovered that reduction of alkali nitrate to nitrite can be complete by iron in the absence of oxygen. Nascent hydrogen, he states, is not involved because iron is capable of drawing to itself the subsidiary valence of the nitrate oxygen atom and consequently of abstracting an atom of oxygen from the nitrate molecule. Baudisch (19) also reported that dextrose and iron are factors in the reduction of tissue nitrate to nitrite.

This discussion shows that the reduction of nitrate to nitrite in the body appears to be a part of a physiological oxidation-reduction system, especially prevalent in liver tissue. A diminution in enzymatic activity of this type may occur in ageing to account for the low nitrite values in blood of the aged, although such evidence has not yet been revealed by investigators interested in geriatrics.

## SUMMARY

1. A method has been proposed for the quantitative determination of nitrite in blood.
2. The blood of the dog, monkey, steer, and man contains approximately 10 gamma of nitrite per 100 cc.
3. The nitrite content of the blood of the dog is refractory to change by dietary modifications.
4. The nitrite content of the blood significantly diminished with age in 170 individuals studied in these experiments.

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# NITRITES

# IX. A FURTHER STUDY OF THE MECHANISM OF THE ACTION OF ORGANIC NITRATES[1]

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Recently it was demonstrated that after erythrol tetranitrate, glyceryl trinitrate and mannitol hexanitrate are heated with sodium hydroxide to bring about hydrolysis they do not retain depressor activity in the dog (1). Isomannide dinitrate is an exception in that it retains this activity after an attempt is made to hydrolyze it by alkali treatment. *In vitro* experiments showed that the latter compound is refractory to hydrolysis and this fact offers a possible explanation for the retention of its depressor activity after such treatment, but the other 3 organic nitrates are readily hydrolyzable by the same chemical means.

As Krantz and Carr (1) point out in their report, there remains the unlikely possibility of the immediate reduction of the intact nitrate ester to the nitrite ester, the latter being responsible for the depressor effect observed. The purpose of this investigation is to shed light on the fate of injected nitrite in the body, the presence or absence of nitrite in the blood after the administration of organic nitrates, and the relationship between blood-nitrite content and depressor response.

METHODS. Dogs under nembutal anesthesia (0.75 cc. of a 4% solution per kilogram), were given sodium nitrite and organic nitrates by intravenous injection. The blood pressure in the carotid artery was measured and recorded by a kymograph in the usual manner. Injections were made into the cannulated saphenous or femoral vein and samples of blood were withdrawn from the exposed contralateral femoral artery. Solutions of erythrol tetranitrate, isomannide dinitrate and glyceryl trinitrate were prepared in concentrations ranging from 1:10,000 to 1:1000 in 20 to 35 per cent alcohol. The usual dosage of the nitrates was 0.75 cc. per kilogram. A quantity of alcohol equivalent to that present in each of the doses the dogs received did not exert any depressor activity. The dosage of sodium nitrite ranged from 0.75 cc of a 1:1000 solution to 0.3 cc. of a 5% solution per kilogram.

RESULTS. *Sodium nitrite.* Intravenous administration to 4 dogs of 0.3 cc. of a 5% sodium nitrite solution per kilogram resulted in an average fall in blood pressure of 35 millimeters of mercury (26% below original) within 1 minute after injection (table 1). The nitrite concentration in the blood at this time was, on the average, 740 gamma per 100 cc., this level decreasing to 184, 107 and 68 gamma after 5 minutes, 15 minutes and 45 minutes, respectively. It is of in-

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[1] The expense of this investigation has been defrayed in part by a grant from the Bressler Research Alumni Fund of the University of Maryland.

[2] The material contained in this paper is part of a thesis submitted by Maurice M. Rath to the faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

terest to note that of the nitrite found at the time (within 1 minute) of depressor activity, 75% had disappeared 5 minutes after injection and only another 15% during the next 40 minutes.

Figure 1 also shows that only an average of 7% of the amount of nitrite injected was recoverable from the blood obtained at the time of the fall in blood pressure, and from that withdrawn 45 minutes after injection less than 1% could be recovered. The number of milligrams of nitrite present in 100 cc. of blood, assuming that all the nitrite remained in the blood stream, was calculated from the estimated blood volume and from the amount of nitrite injected. This theoretical amount was considered 100% of the nitrite theoretically recoverable from the blood instantly after the administration of the drug. The total amount of blood in the dog was calculated on the basis of 97 cc. per kilogram body weight (2). In each case the control value for the blood before administration of the drug was subtracted from the actual nitrite level to obtain "recovered nitrite."

TABLE 1

*Effect of sodium nitrite administered intravenously upon the blood pressure of dogs and the concentration of nitrite in the blood at intervals after injection*

| DOGS | TIME AFTER INJECTION | BLOOD PRESSURE | MILLIMETERS BELOW ORIGINAL | PERCENTAGE FALL | NITRITE PER 100 CC. BLOOD |
|---|---|---|---|---|---|
| | | Average | | | |
| | *min.* | *mm. Hg* | | | *gamma* |
| 4 (total no.) | 0 | 131 | 0 | 0 | 11 |
| | ¼ | 96 | 35 | 26 | 740 |
| | 5 | 98 | 33 | 25 | 184 |
| | 15 | 88 | 43 | 32 | 107 |
| | 45 | 98 | 33 | 25 | 68 |

Evidently the level of the nitrite concentration does not bear a direct relationship to the blood pressure after the initial fall has ensued. Even after most of the injected nitrite had disappeared from the blood stream the blood pressure remained considerably below normal. Figure 1 illustrates graphically the rate of disappearance of the injected nitrite and the relationship between the nitrite and blood pressure levels. The explanation for the disappearance of nitrite has not yet been definitely determined.

Sodium nitrite was intravenously administered to several animals (table 2) in doses so small as to evoke little or no fall in blood pressure. Under the influence of a nitrite level of 90 gamma per 100 cc. of blood there was no depressor effect (dogs numbered 35 and 36). When, however, a 5 to 7% fall in blood pressure occurred the blood-nitrite concentration was relatively high (several hundred gamma per 100 cc. in dogs numbered 32, 33 and 34).

*Organic nitrates.* In concentrations of 1:1000, neither erythrol tetranitrate, isomannide dinitrate nor glyceryl trinitrate increased the blood nitrite level at the time of average blood pressure falls of 44, 22, and 40% respectively. In the case of isomannide dinitrate, a very slight increase (2 to 3 gamma per 100 cc. of blood) was noted about 17 minutes after injection of the drug when the blood pressure had risen appreciably.

Erythrol tetranitrate, in the relatively high concentration of 1:100, effected an average of 50% fall in blood pressure in 4 dogs with no increase in blood nitrite at the time of fall. Five minutes after injection there was an average increase of 9 gamma per 100 cc., but sixty minutes after injection the nitrite level returned to its original value. There were slight increases over normal (ranging from 1 to 18 gamma with an average of 6 gamma per 100 cc.) at the time of fall in blood pressure with isomannide dinitrate and glyceryl trinitrate in 1:100 concentrations. The highest level of nitrite in the blood after injecting any of these organic nitrates was but a very small fraction of the lowest nitrite level observed with a comparable fall in blood pressure with sodium nitrite. Furthermore, the increase in nitrite concentration (table 2) under sufficiently minute doses of sodium nitrite to cause no fall in blood pressure was considerably greater than any increase in nitrite occurring after injection of the organic nitrates with appreciable depression of the blood pressure.

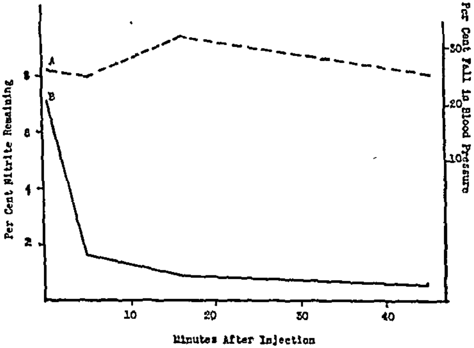

FIG. 1. THE PERCENTAGE FALL IN BLOOD PRESSURE AND RATE OF DISAPPEARANCE OF NITRITE AFTER INTRAVENOUS ADMINISTRATION IN DOGS

Curves A and B show, respectively, the average percentage fall in blood pressure and the percentage of nitrite remaining in the blood stream of 4 dogs following the intravenous injection of 15 milligrams of sodium nitrite per kilogram.

Finally, erythrol tetranitrate and glyceryl trinitrate in small doses of 0.75 cc. of 1:10,000 solutions per kilogram lowered the blood pressure 14% and 41% respectively, with no increase in the blood nitrite over the control value. In several other dogs, erythrol tetranitrate and glyceryl trinitrate in the same dosage produced a fall of over 30 per cent in blood pressure.

The unlikely possibility remained that from this small quantity of organic nitrate sufficient nitrite might have been quickly liberated in the body to effect a hypotensive action, i.e., be liberated and disappear so quickly as to escape detection by the chemical analysis employed. The amount of nitrate contained in 0.75 cc. of a 1:10,000 solution of either erythrol tetranitrate or glyceryl trini-

TABLE 2

*Intravenous administration of sodium nitrite in amounts causing no or little fall in blood pressure in dogs and the concentration of nitrite in the blood at intervals after injection*

| DOGS | TIME AFTER INJECTION | BLOOD PRESSURE | MILLIMETERS BELOW ORIGINAL | PERCENTAGE FALL | NITRITE PER 100 CC. BLOOD |
|---|---|---|---|---|---|
| Dose: 0.75 cc. of 1:100 solution per kilogram | | | | | |
| | | | | | *gamma* |
| 32 | 0 | 104 | 0 | 0 | 10 |
| | 19 secs. | 98 | 6 | 5 | 320 |
| | 5 min. | 84 | 20 | 19 | 150 |
| | 15 min. | 82 | 22 | 21 | 120 |
| | 45 min. | 92 | 12 | 11 | 70 |
| Dose: 0.75 cc. of 1:100 solution per kilogram | | | | | |
| 33 | 0 | 184 | 0 | 0 | 12 |
| | 60 secs. | 170 | 14 | 7 | 1,110 |
| | 3.5 min. | 144 | 40 | 21 | 750 |
| | 5 min. | 150 | 34 | 18 | 500 |
| | 15 min. | 140 | 44 | 23 | 200 |
| | 45 min. | 148 | 38 | 19 | 150 |
| Dose: 0.50 cc. of 1:100 solution per kilogram | | | | | |
| 34 | 0 | 154 | 0 | 0 | 10 |
| | 27 secs. | 148 | 8 | 5 | 1,000 |
| | 5 min. | 150 | 4 | 2.5 | 400 |
| | 15 min. | 150 | 4 | 2.5 | 350 |
| Dose: 0.75 cc. of 1:500 solution per kilogram | | | | | |
| 35 | 0 | 118 | 0 | 0 | 10 |
| | 45 secs. | 118 | 0 | 0 | 90 |
| | 5 min. | 120 | 0 | 0 | 40 |
| Dose: 0.75 cc. of 1:750 solution per kilogram | | | | | |
| 36 | 0 | 124 | 0 | 0 | 5 |
| | 45 secs. | 124 | 0 | 0 | 55 |
| | 7 min. | 116 | 8 | 6 | 27 |
| Dose: 0.75 cc. of 1:1,000 solution per kilogram | | | | | |
| 36* | 0 | 106 | 0 | 0 | 5 |
| | 45 secs. | 106 | 0 | 0 | 33 |
| | 5 min. | 108 | 0 | 0 | 12 |

* Sixty minutes after first injection.

trate is 0.06 milligram. This amount of nitrate administered per kilogram of dog could give rise to 0.045 milligram of nitrite per 100 cc. of blood, *if* 100% of the nitrate were converted. Crandall (3), however, showed that 20% of glyceryl trinitrate injected intravenously into dogs could be recovered from the blood 1 minute after administration of the drug. Hence if 80% of the nitrate were converted and none left in the circulatory system to enter the tissues, a maximum of 0.038 milligram of nitrite per 100 cc. of blood *might* have been produced, if such rapid hydrolysis and reduction were possible. Such a minute amount of nitrite in the blood, however, could not possibly effect a reduction in blood pressure. This is illustrated by the experimental findings with sodium nitrite and also by mathematical deduction. The amount of sodium nitrite theoretically required to place a concentration of 0.038 mgm. of nitrite ion per 100 cc. in the blood stream immediately upon injection is 0.75 cc. of a 1:13,000 solution per kilogram. This theoretical dose is 150th of the actual amount of sodium nitrite required to produce a fall in blood pressure comparable to that effected by erythrol tetranitrate or glyceryl trinitrate in the concentration under discussion. In fact, nitrite levels of the magnitude of 0.038 mgm. (38 gamma) per 100 cc. could not be associated with any fall in blood pressure.

Furthermore, upon the oral administration of these same organic nitrates to dogs in small and massive doses there occurred no augmentation of the blood-nitrite content.

Discussion and summary. Neither intravenous nor oral administration of the organic nitrates, erythrol tetranitrate, isomannide dinitrate and glyceryl trinitrate, results in the ready liberation of the nitrite ion to the blood. The basis for the general opinion that these compounds yield nitrite in the body before they act to lower blood pressure has rested chiefly on the observation of "methemoglobin" formation after their administration (since nitrite produces methemoglobin).

It should be pointed out that the foregoing conclusions are founded on experiments with dogs. There remains the possibility that the reactions of dogs to nitrates differ from those of other animal species. The validity, however, of the earlier reports that methemoglobin was actually produced in man and laboratory animals after nitrate administration is open to question.

The uncertainty of the presence of methemoglobinemia in early reports after nitrate therapy is attributable to the fact that the first specific quantitative test for methemoglobin—that of Van Slyke (4)—was not proposed until 1911. Many of the statements of methemoglobinemia were made because of the presence of cyanosis in subjects receiving nitrites or because of a brown coloration of the blood. Spectroscopic examination of blood for methemoglobin may not have been entirely accurate according to Kobert (5), who stated that the ordinary spectroscopic examination is uncertain when less than 25% of the blood pigment is in the form of methemoglobin. Rabe (6) pointed out inconsistencies in the results reported by different authors from spectroscopic examinations. As for cyanosis, this condition, accompanied by change of part of the hemoglobin into a non-oxygen carrying form, may not be due to methemoglobin formation at all, according to Loeb, Bock and Fitz (7). Furthermore, recent investigations by

Krantz, Carr and collaborators (8) have demonstrated that under full depressor response of isomannide dinitrate, glyceryl trinitrate or erythrol tetranitrate, blood taken from the carotid artery of the dog under ether anesthesia contained no methemoglobin and showed no characteristic spectral band when examined in the Keuffel and Esser Color Analyzer. Tests were conducted at intervals until one hour after injection. *In vitro* experiments were also conducted with 1% dilutions of normal dog blood saturated with erythrol tetranitrate, mannitol hexanitrate and isomannide dinitrate, respectively. The presence of methemoglobin in the blood could not be detected when tested by the Color Analyzer one hour later.

For these reasons we believe that the conclusions of earlier investigators, that nitrite must be formed from nitrates before the blood pressure can be reduced are founded on an insecure basis.

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# THE EFFECT OF CARMINATIVES ON THE EMPTYING TIME OF THE NORMAL HUMAN STOMACH

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Conflicting reports of the action of carminatives on the gastrointestinal tract may be found in textbooks of pharmacology. Meyer and Gottlieb (1) state that the muscular activity of the alimentary tract is increased by carminatives. Sollman (2) suggests that the aromatics and condiments probably act by mild irritation of the gastric mucosa and that the beneficial effects are due to a hyperemia and motor stimulation. In Cushny's textbook (3), however, it is mentioned that volatile oils decrease the movements and tone of the stomach.

Investigators studying the action of carminatives on the gastrointestinal tract also have reported variable results. Heubner and Rieder (4) in 1909 found that the sojourn of food in the stomach was shortened by small but delayed by large doses of stomachics. Morse (5) in 1916 reported that tabasco sauce did not modify the discharge of water from the stomach. In the same year Muirhead and Gerald (6) reported that the motility of isolated segments of the intestine was increased by low concentrations (1:50,000) of volatile oils but decreased with higher concentrations (1:5,000). Gunn (7) in 1920, also working with volatile oils on isolated pieces of the intestine, found that in dilutions of 1:20,000 and up the rate and extent of movement decreased and relaxation resulted. No effect was noted with lesser concentrations. Plant (8) in 1921, working with dogs with Thiry-Vella loops, reported that carminative oils in dilute solutions when placed in the intestinal loop caused an increase in tone and rhythmic contractions. Later Plant and Miller (9) studied the action of a group of volatile oils on the gastrointestinal tract and reported that therapeutic doses of dilute solutions of these oils caused a relaxation of the walls and a decrease in peristaltic contractions of the stomach. This relaxation lasted from five to thirty minutes and in about one-half of the cases was followed by some increase in tone or amplitude of contraction. The tone and contractions of the small intestine, however, were increased. Stross (10) in 1922 working with a number of derivatives of volatile oils found that for the main part they decreased the motility of intestinal strips and the contractions in rings of the stomach of the frog. Armond (11) in 1925 found a paralyzing action of peppermint on the stomach, but a stimulating action on the intestine.

Several observations have been reported on the effect of carminatives and bitters on the intact stomach of man. In 1915 Carlson and his coworkers (12, 13) reported that bitters in the usual therapeutic quantities had no apreciable effect on the hunger mechanism. Indeed these workers reported that these substances in larger than therapeutic quantities produced an inhibition or depression of hunger. In 1920 Miller *et al.* (14) observed that a noticeable delay in gastric emptying was produced following the ingestion of creamy wafers which had a strong peppermint flavor. Sommerfield *et al.* (15), however, using the fluoroscope observed that peppermint increased the amplitude and vigor of gastric contractions. Sapoznik, Arens, Meyer and Necheles (16) in 1935, also using the fluoroscopic method, reported that oil of peppermint in large doses (2 cc.) materially reduced the emptying time of the stomach both in man and in the dog. These workers felt that the contrary results reported by Miller *et al.* could be explained by the fact that a great amount of candy had been eaten which contained a high peppermint content, and further that at times oil of peppermint could produce antiperistalsis. It should be pointed out, however, that the doses of peppermint used by Sapoznik *et al.*, too, were very large ones, in fact about twenty times the therapeutic dose.

From this brief review of the literature it may be seen that variable results have been reported by investigators not only working with isolated segments of the gastrointestinal tract, but also working with the intact stomach. Because of these discordant results and because relatively few well controlled studies have been made of the effect of carminatives on the normal gastric emptying time of man it was considered worthwhile to study the problem further.

METHODS. The subjects used in these investigations were twenty young healthy male adults. The test meal used was the same as that which has been described previously (17). It consisted essentially of 15 gms. of Quaker Farina cooked with water to a volume of 200 cc. Fifty grams of barium sulfate were admixed so that the position of the meal could be determined fluoroscopically. The subjects ate this meal about 9 a.m. No food had been eaten since the preceding evening. The time required for the meal to leave the stomach completely was determined to the nearest fifteen minutes. A number of control observations were made at exactly weekly intervals. The average figure was used for the norm.

Six subjects were given 1 cc. of Fluidextract of Ginger, U.S.P.XI, which had been thoroughly mixed with the test meal. The gastric emptying time was again determined. Five determinations were made on all the subjects save one, on whom four observations were made. The average figure was used to express the results.

The effect of Tincture of Capsicum, U.S.P.XI, was now studied. The same procedure was used as when the effect of ginger was studied. Three of the subjects received 0.5 cc., six of the subjects received 1 cc. Three or four observations were made on most of these subjects; one subject, however, received it five times but two received it only once. As in the case of ginger, the average figure was used.

Oil of Peppermint, U.S.P.XI, in doses of 0.3 cc. was given to six subjects and the same procedure was used as previously outlined. No less than three observations were made on each individual. The average figure was again used to express the results.

RESULTS. The results are shown in tables 1, 2 and 3. It will be noted that fluidextract of ginger produced a statistically significant decrease in gastric emptying time in one individual only. The other five subjects were not significantly affected. Tincture of capsicum also produced a statistically significant decrease in the gastric emptying time of one subject; the remainder of the subjects, however, were relatively unaffected. It is worthy of note that the subject in whom capsicum produced a decrease in gastric emptying time was not the same individual in whom ginger caused a decrease. Furthermore no particular significance is attached to the fact that a significant decrease in gastric emptying occurred in one of the subjects who received a smaller dose (0.5 cc.) of tincture of capsicum, because two other subjects who received a dose of the same size were unaffected.

TABLE 1

*The effect of 1 cc. of fluidextract of ginger on the gastric emptying time*

| SUBJECT NO. | CONTROL | NO. OF TRIALS | AFTER INGESTION OF GINGER | NO. OF TRIALS | DIFFERENCE | STANDARD ERROR |
|---|---|---|---|---|---|---|
| | *hours* | | *hours* | | | |
| 1 | 2.30 | 5 | 2.70 | 5 | +.40 | .31 |
| 2 | 1.50 | 5 | 1.19 | 4 | −.31 | .10* |
| 3 | 2.80 | 5 | 2.25 | 5 | −.55 | .24 |
| 4 | 1.90 | 5 | 2.00 | 5 | +.10 | .37 |
| 5 | 2.25 | 5 | 2 55 | 5 | +.30 | .27 |
| 6 | 1.95 | 5 | 1.90 | 5 | −.05 | .24 |
| Average. ... | 2.12 | 5 | 2 10 | 4.8 | −.02 | .28 |

* Significance was chosen as three times the standard error.

TABLE 2

*The effect of 1cc. of tincture of capsicum on the gastric emptying time*

| SUBJECT NO | CONTROL | NO. OF TRIALS | AFTER INGESTION OF CAPSICUM | NO. OF TRIALS | DIFFERENCE | STANDARD ERROR |
|---|---|---|---|---|---|---|
| | *hours* | | *hours* | | | |
| 7† | 1.92 | 3 | 2.00 | 2 | +.08 | .26 |
| 8† | 2.08 | 3 | 2 56 | 4 | +.48 | .18 |
| 9† | 1.80 | 5 | 1.35 | 5 | −.45 | .08* |
| 7 | 2.33 | 3 | 2.19 | 4 | −.14 | .15 |
| 10 | 1.90 | 5 | 2.88 | 4 | +.98 | .35 |
| 11 | 2.15 | 5 | 1.58 | 3 | −.57 | .22 |
| 12 | 1.75 | 3 | 1.75 | 1 | | |
| 13 | 1 92 | 3 | 1.25 | 1 | | |
| 14 | 2.00 | 3 | 2.44 | 4 | +.44 | .47 |
| Average.... | 1.98 | 3.7 | 2.00 | 3.2 | +.02 | .19 |

* Significance was chosen as three times the standard error.
† These received only 0.5 cc. of the tincture of capsicum.

TABLE 3

*The effect of 0.3 cc. of oil of peppermint on gastric emptying time*

| SUBJECT | CONTROL | NO OF TRIALS | AFTER INGESTION OF OIL OF PEPPERMINT | NO. OF TRIALS | DIFFERENCE | STANDARD ERROR |
|---|---|---|---|---|---|---|
| | *hours* | | *hours* | | | |
| 15 | 2.25 | 5 | 2.31 | 4 | +.06 | .24 |
| 16 | 1.75 | 4 | 1.94 | 4 | +.19 | .12 |
| 17 | 3.30 | 5 | 3.25 | 4 | −.05 | .47 |
| 18 | 2.35 | 5 | 1.88 | 4 | −.47 | .38 |
| 19 | 2.90 | 5 | 2.75 | 3 | −.15 | .30 |
| 20 | 1.75 | 6 | 1.50 | 3 | −.25 | .39 |
| Average | 2.38 | 5 | 2.27 | 3.7 | −.11 | .36 |

The oil of peppermint had no appreciable effect on gastric emptying. One of six subjects showed a decrease of 0.47 hours, but statistically this was not significant.

Discussion. The results show clearly that as far as the effect of carminatives on normal gastric emptying is concerned there may be considerable individual variation. Although gastric emptying time was not significantly affected by fluidextract of ginger in five of six individuals, one subject showed a significant decrease. Virtually the same results were obtained when the effect of capsicum was studied; in this instance the gastric emptying time in seven of eight subjects was unaffected, but that in one subject was significantly decreased.

According to our findings, oil of peppermint in therapeutic doses does not appreciably affect gastric emptying. As previously mentioned, Sapoznik, Arens, Meyer and Necheles (16) reported that large doses of oil of peppermint produced a noticeable acceleration of gastric emptying. These workers made no mention of the effect of small doses. We feel, however, that the action of reasonable therapeutic doses of drugs is more important both to medicine and pharmacology than is the action of massive doses, and in point of fact the results obtained may be quite the opposite (17).

It would seem from the study of these three representative carminatives that the gastric emptying time in the average individual probably is but little affected by ingestion of therapeutic doses of such substances. It is likely, however, that the gastric emptying of some individuals probably would be affected by certain carminatives; in some subjects, moreover, gastric motility would be increased and in others decreased.

It is in order to comment on the mode of action of bitter or irritating substances in general on the gastrointestinal tract. It has been pointed out by Gunn (7) that volatile oils may affect the stomach in one of two ways, namely, either by direct action on the muscle, causing relaxation, or by a reflex arising from irritation of the gastric mucosa causing contraction. The ultimate effect would depend upon the relative strength of these two actions. It is of interest to note that therapeutic doses of tincture of digitalis (18) and of sodium bicarbonate (19) are both capable of speeding up gastric emptying. They probably produce their effect by local irritation of the gastric mucosa.

It has been suggested by Carlson (20) that anything which stimulates the nerve endings in the gastric mucosa may inhibit the gastric hunger mechanism. It is not illogical to assume that the same would apply to the movements of the stomach containing food. It would seem, then, that if carminatives caused considerable irritation of the gastric mucosa some inhibition might ensue rather than any increase in gastric motility.

It has been emphasized that irritating substances do not have the same effect on gastric emptying in all subjects, that is, there is a noticeable individual variation. It is difficult to account for this although one or two factors could be mentioned which might be responsible for this phenomenon. It has been observed that some carminatives may produce antiperistaltic waves in the stomach. The susceptibility of individuals to these antiperistaltic waves as well as the duration of the waves may well vary in different subjects. This could account for the individual variation seen in gastric emptying following the ingestion of certain carminatives. Another explanation which may be offered is that the threshold of irritability of the gastric mucosa varies in different individuals. If we assume that this is possible, it would be expected that individuals would respond differently to the action of carminatives, some showing acceleration of gastric emptying, others inhibition, and still others no effect. This could account, in part at least, for the discordant results found in the literature.

## SUMMARY AND CONCLUSIONS

Fluidextract of Ginger, U.S.P.XI, in 1 cc. doses was given to six subjects in a suitable test meal. One of the six subjects showed a statistically significant decrease in the gastric emptying time as ascertained fluoroscopically. The gastric emptying of the remainder of the group was not significantly affected.

Tincture of Capsicum U.S.P.XI, was given to three subjects in doses of 0.5 cc. and to six subjects in doses of 1 cc. One individual showed a statistically significant decrease in the gastric emptying time; the other subjects were relatively unaffected.

Six subjects were given 0.3 cc. of oil of peppermint, U.S.P.XI. None of the subjects showed either a significant increase or decrease in gastric emptying.

It was concluded that representative carminatives, such as capsicum, ginger and oil of peppermint may influence gastric emptying in certain subjects, but probably the gastric emptying time of the large majority of individuals is not appreciably affected by the ingestion of these substances in moderate doses.

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# STABLE ANESTHETIC SOLUTIONS OF BARBITURIC ACIDS*

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The desire for stable, non-irritating solutions of barbituric acid hypnotic-anesthetics led us to make this study of the many possible solute-solvent combinations. We were particularly interested in obtaining solutions which would not be irritating when injected intramuscularly, and which at the same time would be sufficiently concentrated that any desired degree of hypnosis or anesthesia could be produced by injection of relatively small volumes. We have found at least one such solution which seems suitable for intramuscular use, and several which offer possibilities as intravenous anesthetics.

Clinically it is often desired to administer barbituric acid hypnotics by injection. For this purpose it is generally necessary to prepare an aqueous solution of the sodium salt of the acid. Because of their instability these solutions must be prepared just before use and from sterile components. Most of them decompose appreciably even on standing at 25° for a day. Moreover the solutions are strongly alkaline and are irritating to tissues.

It seemed to us possible that suitable solvents could be found for the relatively stable, almost neutral acid-form of one or more of the clinically familiar shorter-acting barbituric acid derivatives. These acids are so weak (*pK′* generally not very different from 8) (1) and of such stability and low solubility in water that they should cause no irritation of tissues, yet the pH of the tissue fluids is high enough that absorption should not be too slow.

Our first solubility and pharmacological studies were carried out using olive oil as solvent, as this is a well-known vehicle for intramuscular injection of certain drugs other than hypnotics. Because this oil dissolves most barbituric acids only sparingly we subsequently tried the related triacetin and a number of other solvents. In table 1 a summary of the solubilities is given. (These data may be in error by as much as ± 10%.) A number of solvents not in the table were studied: ethyl lactate; cellosolve; butylcellosolve; saturated aqueous solutions of urea, of methylurea, of sym. dimethylurea, of urethane, of acetamide. These often dissolved appreciable amounts of the barbituric acids, but for a reason appearing later in this paper they were set aside as less suitable for intramuscular injections than solvents less soluble in water.

The activity of such solutions depends in part upon the concentration, the rate of absorption (site of administration), the inherent activity and the rate of detoxification of the active substance, and under some circumstances upon the solubility of the solvent in body fluids. We chose as a goal solutions which would contain an intramuscular anesthetic dose in a volume of not more than 0.1 cc. per kilogram of animal (rat, dog, man). Of the barbituric acids in table 1 only a few are both sufficiently soluble and sufficiently active to fulfill this requirement. To satisfy our limit of 0.1 cc. of solution per kilogram of animal a barbituric acid having a solubility of 500 mg. per cc. of solution must produce anesthesia in a dose of 50 mg. per kilogram or lower; this gives an "activity/solubility" ratio $= \frac{\text{mg./kg.}}{\text{mg./cc.}} = 0.1$ cc./kg. or lower. A barbituric acid having an AD 50 of 30 mg./kg. and a solubility in the solvent of only 100 mg. per cc. would require that $\frac{30}{100} = 0.3$ cc. per kilogram be injected. Since solutions of about 30 to 50% were as concentrated as seemed practical, our pharmacological tests were immediately limited to such drugs as have an AD 50 intravenously of 50 mg. per kilogram or lower. (Because intramuscular AD 50 is greater than

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TABLE 1

*Solubility of barbituric acids in various solvents*

| BARBITURIC ACID | SOLVENTS | | | | |
|---|---|---|---|---|---|
| | Formamide | Propylene glycol | Olive oil | Triacetin | Paraldehyde |
| | mg /cc. of solution at 25°C. | | | | |
| *5*,*5*-Diethyl- (Barbital) | | 120 | | 45 | <9 |
| *5*-Ethyl-*5*-*n*-butyl- (Neonal) | | 500 | | 250 | 91 |
| *5*-Ethyl-*5*-isoamyl- (Amytal) | 250 | 125 | | 95 | <40 |
| *5*-Ethyl-*5*-(*1*-methylbutyl)- (Pentobarbital) | | 250 | | 167 | <100 |
| *5*-Ethyl-*5*-*n*-hexyl- (Ortal)) | | 250 | | 145 | 56 |
| *5*-Ethyl-*5*-phenyl- (Phenobarbital) | | 150 | | 125 | 12 |
| *5*-*n*-Propyl-*5*-*n*-amyl- | | | 25 | 115 | 43 |
| *5*-*n*-Butyl-*5*-isoamyl- | | | | 47 | 16 |
| *5*-Isobutyl-*5*-isopropyl- | | | 20 | 115 | 45 |
| *5*-*sec*-Butyl-*5*-*n*-butyl- | | | | 91 | 43 |
| *5*-*sec*-Butyl-*5*-isoamyl- | | | | 144 | 59 |
| *5*-Allyl-*5*-isopropyl- (Alurate) | | 250 | | 167 | 47 |
| *5*-Allyl-*5*-(*1*-methylbutyl)- (Seconal) | >300 | >500 | 200 | 400 | >300 |
| *1*-Methyl-*5*-ethyl-*5*-*n*-butyl- (N-Methyl-neonal) | | 120 | <80 | 333 | 200 |
| *1*-Methyl-*5*-ethyl-*5*-(*1*-methylbutyl) (N-Methyl-pentobarbital) | | | >525 | >667 | >500 |
| *1*-Methyl-*5*-allyl-*5*-isopropyl- (Narconumal) | >500 | 333 | 200 | >500 | >500 |
| *1*-Methyl-*5*-methyl-*5*-cyclohexenyl- (Evipal) | 50–60 | 22 | <30 | 50 | 16 |
| *2*-Thio-*5*-ethyl-*5*-(*1*-methylbutyl) (Pentothal) | 50 | | | 63 | 38 |
| *1*-Ethyl-*5*-allyl-*5*-isopropyl- (N-Ethyl-alurate) | | >500 | >400 | >500 | >500 |
| *1*-*n*-Butyl-*5*,*5*-diethyl- (N-*n*-Butyl-barbital) | | 125 | | 200 | 200 |
| *1*,*3*-Dimethyl-*5*,*5*-diethyl- (N,N′-Dimethyl-barbital) | | | | | >500 |
| *1*,*3*-Dimethyl-*5*-ethyl-*5*-*n*-butyl-(N,N′-Dimethyl-neonal) | | | | | >500 |
| *1*,*3*-Dimethyl-*5*-ethyl-*5*-(*1*-methylbutyl)- (N,N′-Dimethyl-pentobarbital) | | | | | >500 |

or equal to intravenous AD 50 this did not eliminate any derivatives which might be as active as this intramuscularly.)

During the progress of the experiments it became apparent that the rate of absorption from an intramuscular site varied enormously with the nature of the solvent. At the same concentration in the water-soluble solvents (formamide, cellosolve, butylcellosolve, propylene glycol) the amount of a short-acting solute equivalent to AD 50 was always much greater than the AD 50 when dissolved in the solvents of low water solubility. (See table 2.) The water-soluble solvents are probably absorbed rapidly, leaving the relatively insoluble solute to be absorbed slowly.

TABLE 2

*Anesthetic activity of solutions of barbituric acids*

| BARBITURIC ACID | SOLVENT | CONCENTRATION MG./CC. OF SOLUTION | INTRAMUSCULAR AD 50 MG./KG.* |
|---|---|---|---|
| *5*-Allyl-*5*-(*1*-methylbutyl)-(Acid form of Seconal) | Propylene glycol | 300 | >50 |
| | Formamide | 300 | >50 |
| | Cellosolve | 300 | >30 |
| | Butylcellosolve | 300 | >30 |
| | Triacetin | 300 | 30 |
| | Olive oil | 250 | 30 |
| | Paraldehyde | 300 | 30 |
| | Aqueous (Na salt) | 100 | 33 |
| *1*-Methyl-*5*-allyl-*5*-isopropyl-(Narconumal) | Paraldehyde | 300 | 50 |
| *1*-Methyl-*5*-ethyl-*5*-*sec*-amyl- (N-Methyl-pentobarbital) | Paraldehyde | 300 | 60 |

* These data were obtained by injection of the 30 per cent solutions into the thigh muscles of male rats. The volume of solution injected was usually somewhat greater than 0.020 cc. and the error of measurement of this volume was in general not greater than ±0.001 cc. Syringes were calibrated. Ten male rats were used on each dose. A rat was considered anesthetized if on forceful and repeated pinching of the tail he could not attain an upright position. The values of AD 50 were estimated by interpolation, and although they may be in error by as much as ± 15 per cent they are sufficiently accurate for the purposes of this study.

In table 2 are tabulated our data on the anesthetic activity of all of the more active solutions. All those solutions of table 1 which had an AD 50 (intramuscularly) of 0.1 cc./kg. or less, and another of lower activity is included.

It is perhaps surprising to find that so few of our original group of solutions answer our requirements as intramuscular anesthetics. The large decrease in effectiveness of solutes in the water-soluble solvents led us to study further only the solutions in the solvents of relatively low water-solubility, namely, olive oil, triacetin, paraldehyde. (Tripropionin and tributyrin are also good solvents.) Of these, paraldehyde and olive oil are well known clinically. The pharmacology of triacetin (and of tripropionin and tributyrin) is apparently unknown.

Paraldehyde offers these important advantages over olive oil: it is a stable, easily available, chemically pure substance; it dissolves many barbituric acids to a much greater extent; it can be administered intravenously, as well as intramuscularly, rectally or orally. We therefore chose to confine our further investigations to barbituric acids dissolved in paraldehyde.

We tested the stability of the 30% solution of *5*-allyl-*5*-(*1*-methylbutyl)-barbituric acid in paraldehyde by heating it at 120 ± 2°C. under a mercury trap for 4 hours. After cooling, the volume decrease was insignificant and the anesthetic activity of the solution had not changed appreciably. This indicated that this barbituric acid is stable and does not catalyse to any appreciable extent the depolymerization of paraldehyde to acetaldehyde. This stability allows sterilization of the solution by heat.

The solutions in paraldehyde which seem to us to be of particular interest are those of *5*-allyl-*5*-(*1*-methylbutyl)-barbituric acid, (*A*), *1*-Methyl-*5*-ethyl-*5*-(*1*-methylbutyl)-barbituric acid, (*B*), and *1*-Methyl-*5*-allyl-*5*-isopropyl-barbituric acid, (*C*). (There are no doubt many closely related derivatives which if they had been available would be in this group.) Of these *A*, the acid form of "seconal," is well known clinically as a very active hypnotic (anesthetic) having a somewhat shorter duration of action than pentobarbital (nembutal). *B* is apparently not known clinically but has been studied in the laboratory (2). *C* (narconumal) is clinically well known in Europe as a short-acting anesthetic; it has been used in several thousand intravenous anesthesias (3). *A* in paraldehyde gives perhaps the optimum combination of inherent activity and rate of detoxification for intramuscular hypnosis or anesthesia. All barbituric acids we have tested which are detoxified less rapidly than *A* have too high an activity/solubility ratio to suit our purpose. Most of the derivatives which are more active on the basis of intravenous dosage are detoxified at such a high rate that the "intramuscular activity"/solubility ratios are too high.

The paraldehyde solution of *A* has been administered intramuscularly to a large number of dogs with results similar to those in rats. The rate of onset of anesthesia after a single injection is about the same in the two animals, usually 20 to 40 minutes with minimal doses. A decrease in this time of onset in dogs and men was attained by injecting portions of the total dose in as many as 6 or 8 intramuscular sites. The amount of drug required to produce anesthesia was also reduced by this multiple injection procedure, but still was greater than the intravenous dose.

Compounds *B* and *C* were not tried extensively in dogs by the intramuscular route because of the larger doses required. (See table 2.) Intravenously however they are much more effective, and we have used paraldehyde solutions of these substances, as well as of *A*, for the production of surgical anesthesia by intravenous injection. By this route these solutions produce effects very like those caused by the injection of the common aqueous solutions of the sodium salts. An injection at a moderately slow rate can be made to produce (in dogs) deep anesthesia (abolition of the corneal, not the wink reflex) without undue depression of respiration or blood pressure. As is the case with all barbiturates, a too rapid injection of an anesthetic dose may produce a fall in blood pressure and respiratory arrest. The dosage and duration with *A* are somewhat less dependent than with *B* and *C* upon the rate of injection. The amounts of all three solutions required to produce this stage of anesthesia in dogs are approximately the same, about 0.05 cc. intravenously per kilogram (300 mg. barbituric acid per cc. of solution).

With the solution of *A* dogs have been maintained in the stage of deep anesthesia mentioned above for 4 to 5 hours by repeated intravenous injections of some 2 to 4 mg./kg./hour, as needed. The duration of the deep anesthesia produced by the initial dose (15 mg./kg. during 5 minutes) varied from 20 to 90 minutes. In similar experiments with paraldehyde solutions of compounds *B* and *C* the initial dose necessary to produce the deep anesthesia varied with the rate of injection, from about 15 to 25 mg./kg. during 2 to 5 minutes. The duration of this anesthesia was about 5 to 15 minutes, and to maintain it for longer periods it was necessary to inject additional amounts of these solutions somewhat more frequently than was the case with the solution of *A*.

All of the dogs recovered without signs of ill effects.

Clinically we have tried only the paraldehyde solution of *A*, intramuscularly in hypnotic doses. In some 30 patients doses up to 5 mg./kg. (0.017 cc./kg.) have been injected in the deltoid or gluteal muscles. The hypnosis from this dose varied from pronounced to slight in various individuals. In no case was a patient discomforted by the injections, nor has there been any sign of irritation or injury to tissue.

We have not undertaken the administration to humans of larger doses for the production of deep hypnosis or of anesthesia. It is quite possible that this solution would be useful in the capacity of a basal anesthetic administered by the convenient intramuscular route, or for the production of full surgical anesthesia by intravenous injection. Clinical trials along these lines are being carried out.

## SUMMARY

We have found a number of solvents which seem to be suitable for the administration of the water-insoluble acid-form of certain barbituric acid derivatives. The most versatile of these solvents are paraldehyde and triacetin. Because we do not know the pharmacology of triacetin, we have concentrated our present studies on solutions in paraldehyde.

A 30% solution in paraldehyde of *5*-allyl-*5*-(*1*-methylbutyl)-barbituric acid seems suitable for the production of any desired degree of hypnosis or anesthesia by intramuscular or intravenous injection. This solution has these advantages over the commonly used aqueous alkaline solutions of barbituric acids: it can be sterilized by heat and remains stable for a long time; it is so slightly irritating that anesthetic doses can be injected intramuscularly without discomfort; the anesthetic dose is contained in a relatively small volume.

For intravenous injection it is possible that corresponding solutions of *1*-methyl-*5*-allyl-*5*-isopropyl-barbituric acid (narconumal) and of *1*-methyl-*5*-ethyl-*5*-

(*l*-methylbutyl)-barbituric acid would also be suitable for the production of relatively short anesthesia, to be lengthened if desired by supplemental injections.

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# STUDIES OF THE INFLUENCE OF PROSTIGMINE[1] ON MORPHINE ADDICTION

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Attempts to reduce the incidence of addiction resulting from the therapeutic use of morphine fall into four categories: *1.* Alteration of the morphine molecule (1, 2); *2.* Synthetic Substitutes (1, 3); *3.* Potentiation of morphine action; *4.* Administration of morphine in minimally effective amounts (4, 5).

While each approach has good features, as yet none has been entirely successful. Recently, Slaughter and his associates have undertaken a new approach in the third category based on the premise that morphine acts in a cholinergic manner. They have studied the effects of administering known cholinergic drugs (eserine, prostigmine) with morphine with the view that certain actions of morphine would be potentiated thereby. Eserine was found to potentiate the toxicity of morphine, and its action on the intestine and on blood pressure (6, 7). Subsequently, it was reported that prostigmine potentiates the analgetic effect of morphine, and that atropine "materially antagonizes" this effect (8, 9).

Their studies on dogs showed that no tolerance or physical dependence developed to prostigmine *per se* over a period of 9 months. When morphine (20 mgm./kgm.) and prostigmine (0.1 mgm./kgm.) were given together, no physical dependence developed; however, the dogs died within 6 weeks (10). Animals receiving 10 mgm./kgm. morphine with 0.1 mgm./kgm. prostigmine daily became only partially tolerant to morphine in 5 months. In a subsequent paper it was reported that after physical dependence had been established to morphine (dogs) and then supported with morphine and prostigmine, the abstinence syndrome which followed withdrawal was less severe than that of morphine alone (11).

Slaughter, Parsons, and Munal reported their clinical experience with this combination in 1940 (12): 8 mgm. morphine and 0.5 mgm. prostigmine provided "excellent relief" from various types of clinical pain, with no untoward effects and less constipation than with morphine alone.

The validity of the basic premise that morphine acts in a cholinergic manner *in man* is open to question since Williams (13) found no significant change in serum cholinesterase activity after doses of morphine up to 100 mgm. On the other hand, Andrews (14) found that *post-addicts* who were refractory to the pain threshold raising effect of 20 mgm. morphine, did show some increase when this amount of morphine was given with 1 mgm. prostigmine. Himmelsbach and Andrews (15) have shown that the effect of 10 mgm. morphine given with 1 mgm. prostigmine 30 hours after withdrawal had no significantly greater ameliorative effect on the morphine abstinence syndrome than 10 mgm. morphine *per se*, and that 1 mgm. prostigmine alone did not affect appreciably the abstinence syndrome intensity.

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[1] Prostigmine methylsulfate was furnished by Hoffmann-La Roche Inc.

[2] Passed Assistant Surgeon.

[3] Biological Chemist.

[4] Research Psychologist.

[5] Surgeon.

Since Slaughter's results suggest that prostigmine (selectively) potentiates the analgetic action of morphine, while not increasing, or perhaps reducing, its liability to cause addiction, studies were undertaken of the effect of prostigmine on morphine addiction in man.

TABLE 1

*Point system for measuring abstinence syndrome intensity by the day (D) or by the hour (H)*

| SIGNS | (D) BY DAY | | (H) BY HOUR | |
|---|---|---|---|---|
| | Points | Limit | Points | Limit |
| Yawning | 1 | 1 | 1 | 1 |
| Lacrimation | 1 | 1 | 1 | 1 |
| Rhinorrhea | 1 | 1 | 1 | 1 |
| Perspiration | 1 | 1 | 1 | 1 |
| Mydriasis | 3 | 3 | 3 | 3 |
| Tremor | 3 | 3 | 3 | 3 |
| Gooseflesh | 3 | 3 | 3 | 3 |
| Anorexia (40 per cent decrease in caloric intake) | 3 | 3 | | |
| Restlessness | 5 | 5 | 5 | 5 |
| Emesis (each spell) | 5 | | 5 | 5 |
| Fever (for each 0.1°C rise over mean addiction level) | 1 | | 1 | 10 |
| Hyperpnoea (for each resp./min rise over mean addiction level) | 1 | | 1 | 10 |
| Rise in A.M. Systolic B.P. (for each 2 mm. Hg over mean addiction level) | 1 | 15 | 1 | 10 |
| Weight loss (A.M.) (for each lb. from last day of addition) | 1 | | | |

Total abstinence syndrome intensity per day or per hour is the sum of the points scored in the (D) or (H) columns respectively, with due attention to the limits.

THE EFFECT OF PROSTIGMINE ON READDICTION TO MORPHINE. In order to learn whether or not prostigmine prevents or modifies the development of physical dependence to morphine, studies were made by a direct approach wherein this combination of drugs was given regularly over a period of time sufficient for physical dependence to develop to morphine alone. Such studies were made on prisoner patients (former addicts who volunteered for the study) with sentences of sufficient length to permit of recovery prior to release. The fact that they were former addicts might have influenced the ease with which physical dependence developed, but there is no reason to believe that this would permit physical dependence to occur to a combination non-addictive to normal persons.

To learn whether or not prostigmine *prevents* the development of physical dependence to morphine, these drugs were administered to two patients (Nos. 317 and 319) for two months. All medication was administered subcutaneously in equally divided doses four times daily; morphine in progressive increments to 180–200 mgm. per day, and prostigmine, 1 or 2 mgm. each day. At the end of the first month both drugs were withheld for 19 hours. Observations for signs of abstinence (table 1) were made on withholding the drugs after one month of administration, and following withdrawal at the end of the second month. After recovery the study was repeated on one of these patients (No. 317) using morphine alone in identical dosage.

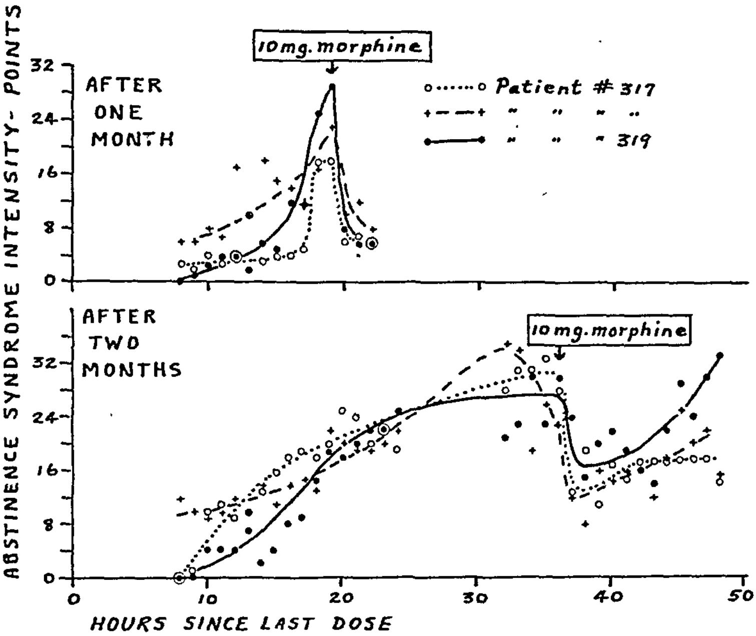
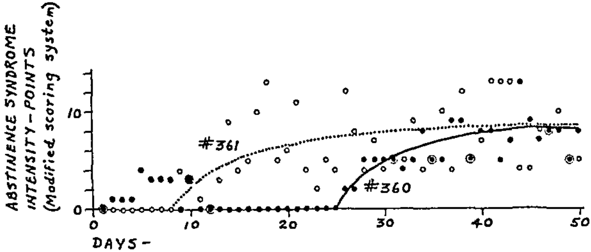


Fig. 1. Upper Graph Shows Development of Abstinence Syndrome on Withholding Morphine (+— —+) and Morphine and Prostigmine (O......O, ●——●) for 19 Hours, after one Month of Administration, and the Effect of 10 mg. Morphine Sulfate.

Lower graph shows the abstinence syndromes of the same patients following withdrawal of drugs after two months of administration, and the effect of 10 mg. morphine at the 36th hour of abstinence.

It was found that a typical abstinence syndrome occurred in each instance following withdrawal (fig. 1). No remarkable differences were noticed between the abstinence syndromes of the patients who received prostigmine with morphine and that of the patient who later served as his own control. The effect of 10 mgm. of morphine was to reduce the intensity of the abstinence syndrome in each instance, thus establishing the validity of the physical dependence.

To learn whether or not prostigmine *modifies* the development of physical dependence on morphine, the former drug was administered in doses of 0.5 mgm. four times daily to one patient (No. 360) of a pair who received morphine in ascending doses for five days, then 200 mgm. once daily (at 10 a.m.) for 50 days.



[illegible] SYNDROME INTENSITY EXHIBITED 20 TO 24 HOURS [illegible] RPHINE BY PATIENT No. 360 (0 5 MG. PROSTIGMINE [illegible] (0.5 ML. 0.85% SOL. NaCl FOUR TIMES DAILY).

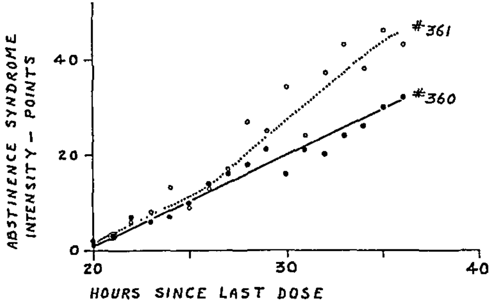


FIG. 3. A PORTION OF THE ABSTINENCE SYNDROMES (20TH TO 36TH HOUR) OF TWO PATIENTS WHO WERE GIVEN 200 MG. MORPHINE ONCE DAILY FOR 50 CONSECUTIVE DAYS

No. 360 was given 0.5 mg. prostigmin q i d. in addition to morphine, and No. 361 was given 0.5 ml. 0.85% sol. NaCl q.i.d.

The other (control) patient (No. 361) was given 0.5 cc. of 0.85% NaCl q.i.d. instead of prostigmine. Observations for signs of withdrawal were made hourly every day from 6 a.m. to noon.[6] The patient who received prostigmine, in addition to morphine, began showing definite signs of abstinence between 6 and 10 a.m. on the 26th day,[7] whereas unmistakable withdrawal phenomena first appeared in the control patient on the 9th day (fig. 2). The abstinence syndrome which followed withdrawal was slightly more severe in the control than in the patient who had been given prostigmine in addition to morphine (fig. 3), but both patients became very ill and required a reduction type of withdrawal treatment.

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[6] In scoring the abstinence syndrome on patients No. 360 and No. 361 changes in temperature, respiration, and blood pressure were not included since these patients were at no time "stable".

EFFECT OF PROSTIGMINE ON CERTAIN PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF MORPHINE ADDICTION. Body temperature, respiration, pulse rate, blood pressure, weight, and caloric intake were depressed during the process of addiction to morphine and prostigmine in patients Nos. 317, 319, and 360. The same changes occurred in patient No. 361 and are usually seen in patients during the process of re-addiction to morphine alone. In patient No. 317 the extent of the depressions in these measures was slightly greater with the combination than with morphine alone.

Hematocrit, hemoglobin, specific gravity of whole blood and plasma, water content of whole blood and plasma, sedimentation, serum viscosity, blood volume, and extracellular water were studied in these patients. The changes in these measures which have been found to occur in addiction to morphine alone (16) occurred also in addiction to morphine and prostigmine. The change in blood volume was not so great as that usually found in uncomplicated morphine addiction, but the effect of withdrawal of morphine-prostigmine on blood volume was somewhat greater than that of morphine alone. It has been shown that serum cholinesterase activity becomes depressed following withdrawal of morphine from addicted patients (13). Studies of serum cholinesterase activity of patients Nos. 317, 319, and 360, gave results in accord with findings on patients addicted to morphine alone.

THE EFFECT OF PROSTIGMINE ON MORPHINE EXCRETION. On alternate weeks 24 hour samples of urine were collected on patients Nos. 317, 319, and 360 and analyzed for morphine (17). The amounts of both free and total morphine excreted increased with the dose of morphine, while the percentage excreted decreased slightly. The ratios of bound to free morphine were of the same order as those found in addicts receiving morphine alone in the same dose (18).

EFFECT OF MORPHINE-PROSTIGMINE ADDICTION ON THE ELECTRODERMAL RESPONSE TO WORD STIMULI. Electrodermal responses to word stimuli were recorded on the Darrow photopolygraph on patients Nos. 317,[8] 319, 360 and 361. The responses (resistance decrease in ohms) were measured from the skin resistance level before each word to the maximum response. The average amplitude of response to the test words was determined before, during, and 30 days after addiction. The results showed reductions in amplitude of electrodermal responses during addiction to morphine-prostigmine similar to those found during addiction to morphine alone (19).

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[7] Perspiration and gooseflesh, considered to have been caused by prostigmine, were noted in patient No. 360 for the first week of its administration.

[8] Tests on patient No. 317 were made in connection with morphine-prostigmine addiction, but not morphine alone.

DISCUSSION. If prostigmine potentiates morphine analgesia in man without increasing the addiction liability of morphine, the use of this combination in the practice of medicine in lieu of morphine alone should operate to reduce the incidence of clinical addiction. Our results indicate that prostigmine does not prevent the development of physical dependence on morphine. Furthermore, conclusive proof is lacking that clinical analgesia is improved by this combination. Although addiction liability is difficult to estimate quantitatively, in general it seems to parallel analgetic power in the morphine series of drugs; e.g. codeine vs. morphine (2). Hence, it would seem that if prostigmine potentiates morphine action, this effect must be selective, for these results do not indicate increased physical dependence action.

While the results reported by Andrews (14) suggest some potentiation of the pain-threshold-raising action of morphine by prostigmine in post-addicts, the relation of this effect to clinical analgesia is still obscure (20). Nor are the results of Slaughter, Parsons, and Munal (12) conclusive in this respect, for they did not establish the fact that the dose of morphine (8 mgm.) employed with prostigmine was ineffective when given alone. Since Lee's studies (5) indicate that 8 to 10 mgm. morphine provides adequate clinical analgesia, it would seem inadvisable to consider that prostigmine potentiates morphine analgesia in man until it is demonstrated that a uniformly ineffective dose of morphine consistently relieves pain when administered with prostigmine.

The experiment carried out to ascertain whether or not prostigmine modifies the development of physical dependence on morphine is inconclusive, for while the onset of appearance of abstinence signs was delayed in the patient receiving prostigmine it is not clear whether this was caused by a difference in the men, the discomfort caused by prostigmine, or a reduction in the addictive action of morphine by prostigmine. Since the patients receiving prostigmine regularly over protracted periods were uncomfortable (nervous and irritable), and since it has been shown that other discomfort (pain) may reduce the addiction liability of morphine (4), this seems the more plausible explanation. In any event it would be difficult to assign clinical importance to the delay encountered under these experimental conditions. Further studies of modification might be indicated by wider clinical experience with the combination.

## CONCLUSION

Prostigmine does not prevent the reproduction of physical dependence on morphine in man; on the other hand it does not appear to potentiate the physical dependence action of morphine.

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addition to morphine, began showing definite signs of abstinence between 6 and 10 a.m. on the 26th day,[7] whereas unmistakable withdrawal phenomena first appeared in the control patient on the 9th day (fig. 2). The abstinence syndrome which followed withdrawal was slightly more severe in the control than in the patient who had been given prostigmine in addition to morphine (fig. 3), but both patients became very ill and required a reduction type of withdrawal treatment.

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# EFFECT OF QUININE UPON THE COURSE OF FOWL-POX INFECTION IN THE CHICK[1]

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Although the use of chemotherapeutic agents against many types of bacterial infections, both in animal and in man, has met with very great success, the case is very different in diseases of virus origin. In lymphopathia venereum (1), lymphocytic choriomeningitis (2), trachoma (3) and smallpox (4) the sulfonamides have been reported to have altered the course of the infection in man. In two of these the beneficial effect may be attributed in part to the action of the drug upon secondary infective agents rather than upon the virus itself. In lymphopathia venereum, the only virus disease produced experimentally, the course of which has been markedly altered by chemotherapy, McKee *et al.* (5) found that in two groups of 13 mice each treated with sulfathiazole and sulfapyridine all survived, but that only 11 of 30 control mice lived; Palmer *et al.* (6) found that mice treated with sulfanilamide or sulfanilylguanidine survived an intracerebral inoculation of lymphopathia venereum, but that after the administration of the drug was discontinued mild signs of infection were observed in some of the treated mice, and that all of the treated mice developed complete immunity against reinfection with lymphopathia venereum. They concluded that the drugs had protected the mice from the disease but had not accomplished an *in vivo* sterilization or attenuation of the virus.

In this laboratory attempts to alter the course of contagious epithelioma (fowl-pox) infection in chicks with various members of the sulfonamide group of compounds as well as with certain other substances met with complete failure, but recent experiments with quinine against fowl-pox produced such striking changes in the course of the infection that it seemed worth while to report them here.

The lesion produced by the fowl-pox virus is similar in two important characteristics to those lesions produced by variola and vaccinia, in that in all three the same tissue, epithelial, is involved and intracellular inclusion bodies are produced. In contrast to the necrotizing process in variola and vaccinia lesions, in fowl-pox there is mainly an hyperplasia of the epithelial elements of the skin.

The fowl-pox infection, as produced in the laboratory by the technic used, does not cause death of the chick, but the lesion that develops in 3–6 days after inoculation is so characteristic in its gross appearance that death is not necessary for the determination of positive infection.

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[1] The funds for this investigation were kindly given by the Mallinckrodt Chemical Works.

EXPERIMENTAL METHODS. *a. Preparation of inoculum and mode of inoculation.*[2] The stock virus for the chick inoculation was grown on the chorioallantoic membrane of the chick embryo, as described by Woodruff and Goodpasture (7). Scrapings from the membrane were macerated or ground with carborundum until a homogeneous paste was made, whereupon the mixture was diluted with distilled water to 1:100 and then centrifuged; the supernatant fluid was used or further diluted immediately before inoculation. In the experiments to be reported below, the final dilutions used were 1:100 and 1:1,000 because the membranes had been in storage for 3 months; however, fresh membranes may be diluted to 1:100,000 and still produce typical lesions. The down, over an area of about 1.5 x 2.5 cm., was plucked from the top of the head of the chick and this area was inoculated immediately by spreading about .02 cc. of the inoculum over the plucked area.

*b. Chick—the experimental animal.* One-day-old chicks, obtained from a commercial hatchery, were placed on the stock diet (Purina Layena) for 10–14 days before use at which time they weighed about 70 grams and were consuming about 10 grams of food per day.

*c. Drug-diet mixtures.* The basis of the diet during the period of administration of the drug to the chick consisted of the residue of the stock diet after shaking through a fine sieve to remove the major part of the bran. To this sifted residue quinine bisulfate was added in quantities to make a 1%, 1.5% and 2% mixture. The chicks were placed upon the drug diet 48 hours before inoculation, and were maintained on this mixture for 14 days following the inoculation, after which time they were placed on the original stock diet and observed until the lesions were completely healed. The diet of the control chicks was the same as the treated except for the lack of quinine.

*d. Record of observations.* The chicks were weighed 48 hours before inoculation, at the time of inoculation, and at regular intervals throughout the experimental period. Each chick was examined daily for the presence and progress of growth of lesions, and three from each group were selected for photographs at frequent intervals throughout the test period. Photographs were made from the third postinoculation day. Three chicks from the control groups were selected because the lesions were typical of the whole group, whereas the chicks from the treated groups were selected by chance because no lesions were present when they were selected.

RESULTS. A summary of results is given in Table 1 but a few comments will help to clarify the picture.

*Inoculum 1:100.* No lesions were visible 48 hours after the inoculation in the control or treated groups, but by 72 hours all of the control chicks showed evidence of typical infection with one chick having a solitary nodule, but in the other 12 chicks multiple lesions were present. There were no gross lesions on the treated chicks at 72 hours, but by 96 hours all but 5 of the treated chicks were positive. The lesions on the treated groups were definitely retarded in their onset of development and growth until the sixth or seventh day, after which time they presented lesions very similar to the untreated control. Pictures of the chicks, from the third to the twelfth postinoculation days, are shown in figure 1; the lesions healed gradually by the twenty-first to the twenty-eighth day.

*Inoculum 1:1,000.* There were no lesions visible in the chicks inoculated with a 1:1,000 dilution until 4 days after inoculation at which time 7 of 12 controls and only 1 of 26 treated had gross lesions. It was only on the seventh day that a majority of the chicks on the 1.5% and 2% quinine diet showed lesions and in these the lesions were smaller than in the non-treated control chicks. Serial

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[2] We wish to thank Dr. G. John Buddingh for our original supply of the virus.

TABLE 1

*The effect of quinine upon the development of the fowl-pox lesion in chicks 3–12 days after inoculation*

| DIET | DAYS AFTER INOCULATION | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | | 4 | | 5 | | 6 | | 7 | | 9 | | 12 | |
| | No chicks | Lesion | No. chicks | Lesion | No. chicks | Lesion | No. chicks | Lesion | No. chicks | Lesion | No chicks | Lesion | No. chicks | Lesion |
| Inoculum 1:100 | | | | | | | | | | | | | | |
| Control | 8 | ++ | 9 | +++ | 10 | +++ | 9 | +++ | 9 | +++ | 11 | +++ | 11 | +++ |
| | 4 | + | 2 | ++ | 2 | ++ | 1 | ++ | 2 | ++ | | | | |
| | 1 | 1N | 1 | + | 1 | + | 1 | + | | | | | | |
| | | | 1 | 1N | | | | | | | | | | |
| 1.0% | 9 | 0 | 1 | ++ | 3 | +++ | 7 | +++ | 8 | +++ | 8 | +++ | 7 | +++ |
| | | | 3 | + | 3 | ++ | 1 | ++ | 1 | ++ | 1 | ++ | 2 | ++ |
| | | | 3 | 2-3N | 2 | + | 1 | + | | | | | | |
| | | | 2 | 0 | 1 | 2N | | | | | | | | |
| 1.5% | 8 | 0 | 1 | ++ | 3 | +++ | 4 | +++ | 4 | +++ | 8 | +++ | 8 | +++ |
| | | | 4 | + | 2 | ++ | 2 | ++ | 4 | ++ | | | | |
| | | | 2 | 2N | 3 | + | 2 | + | | | | | | |
| | | | 1 | 0 | | | | | | | | | | |
| 2 0% | 9 | 0 | 1 | ++ | 1 | +++ | 2 | +++ | 3 | +++ | 5 | +++ | 5 | +++ |
| | | | 3 | + | 1 | ++ | 1 | ++ | 3 | ++ | 1 | ++ | 1 | dead |
| | | | 3 | 1-2N | 2 | + | 3 | + | 1 | dead | | | | |
| | | | 2 | 0 | 2 | 4-5N | | | | | | | | |
| | | | | | 2 | 0 | | | | | | | | |
| Inoculum 1:1,000 | | | | | | | | | | | | | | |
| Control | 12 | 0 | 1 | + | 1 | + | 2 | ++ | 2 | ++ | 2 | +++ | 3 | +++ |
| | | | 6 | 1-2N | 11 | 1-4N | 1 | + | 1 | + | 4 | ++ | 4 | ++ |
| | | | 5 | 0 | | | 9 | 1-5N | 9 | 2-9N | 2 | + | 5 | 2-7N |
| | | | | | | | | | | | 4 | 2-7N | | |
| 1 0% | 10 | 0 | 1 | 2N | 6 | 1-5N | 7 | 1-5N | 1 | + | 1 | +++ | 1 | +++ |
| | | | 9 | 0 | 4 | 0 | 3 | 0 | 8 | 1-8N | 2 | ++ | 3 | ++ |
| | | | | | | | | | 1 | 0 | 7 | 3-4N | 6 | 3-4N |
| 1.5% | 8 | 0 | 8 | 0 | 3 | 1-6N | 5 | 1-6N | 6 | 1-6N | 7 | 2-7N | 1 | ++ |
| | | | | | 5 | 0 | 3 | 0 | 1 | 0 | | | 1 | + |
| | | | | | | | | | 1 | dead | | | 5 | 4-7N |
| 2 0% | 8 | 0 | 8 | 0 | 1 | 1N | 3 | 1-4N | 6 | 1-4N | 2 | + | 2 | ++ |
| | | | | | 7 | 0 | 5 | 0 | 2 | 0 | 5 | 1-6N | 2 | + |
| | | | | | | | | | | | 1 | dead | 3 | 2-4N |

+++ = Confluent lesion covering over 75% of the plucked area.
++ = Confluent lesion covering over 50% of the plucked area.
+ = Confluent lesion covering about 30% of the plucked area.
1-9N = Number of discrete nodules on plucked area.
0 = No lesion.

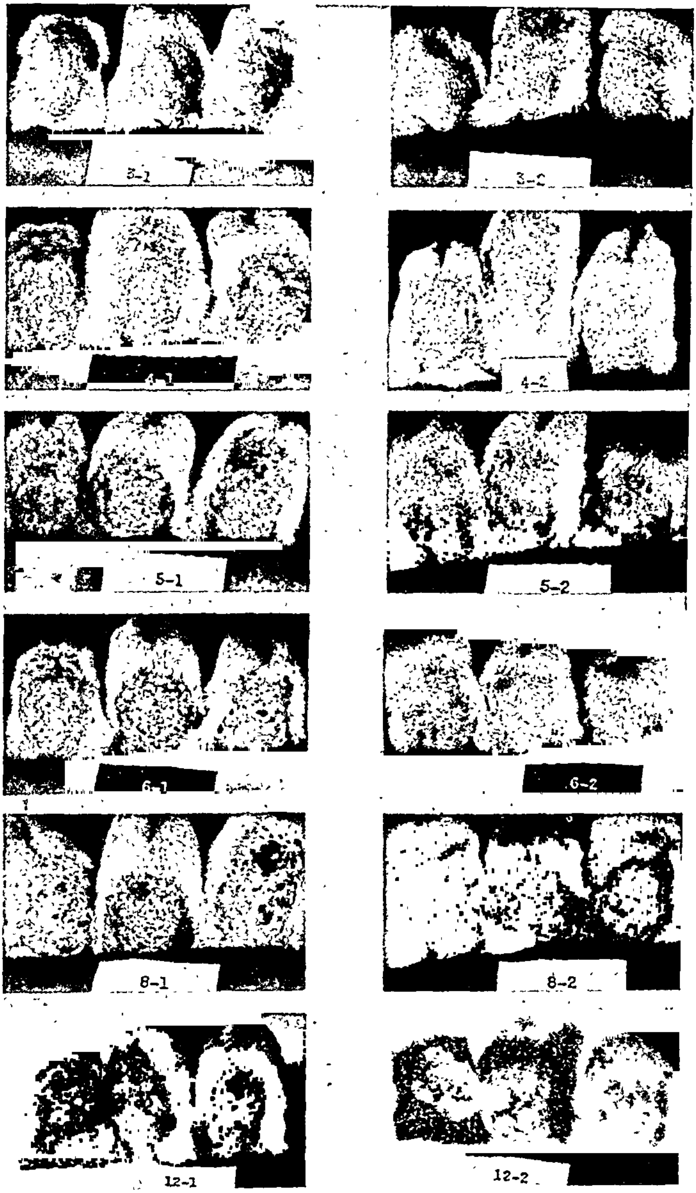

Fig. 1. Photographs of the heads of chicks inoculated with fowl-pox. Dilution 1:100. 3-1, Control chicks on third postinoculation day; 3-2, Chicks on a diet containing 1.0% quinine bisulfate; 3-3. Chicks on a diet containing 1.5% quinine bisulfate; 3-4, Chicks on a diet containing 2.0% quinine bisulfate.

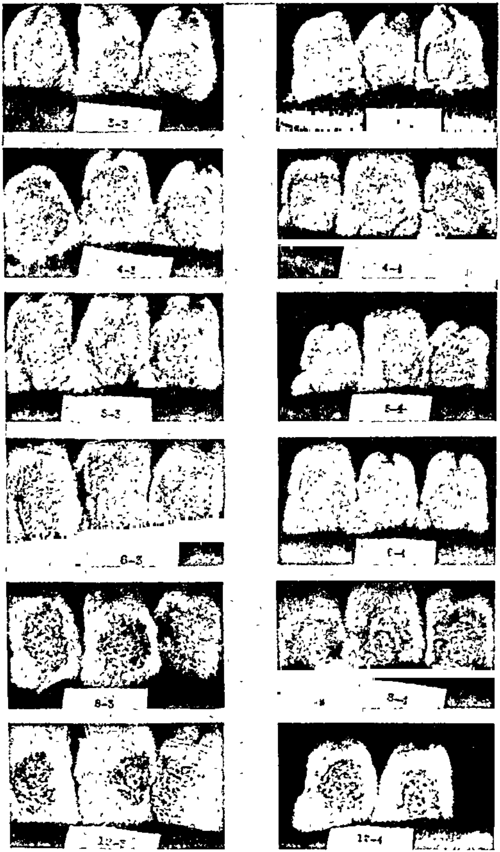
3-3
4-3
4-4
5-3
5-4
6-3
6-4
8-3
8-4
12-3
12-4

FIG. 1—*Continued*

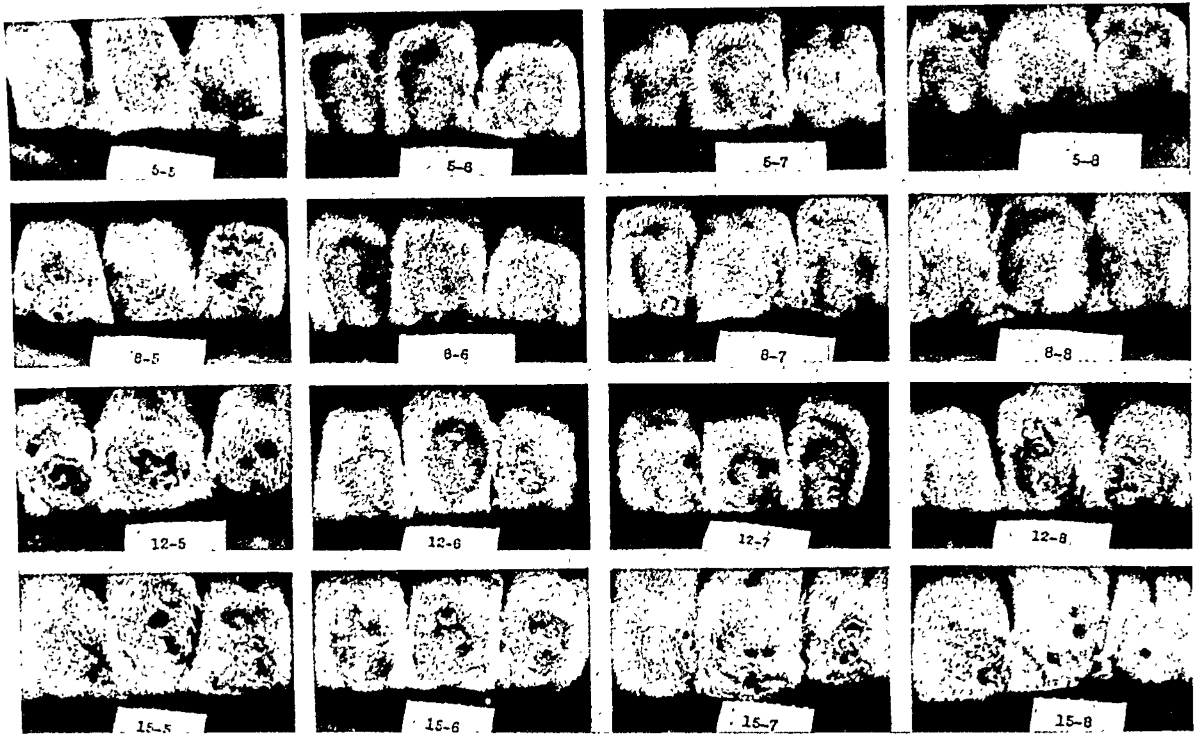
5-5
5-6
5-7
5-8
8-5
8-6
8-7
8-8
12-5
12-6
12-7
12-8
15-5
15-6
15-7
15-8

FIG. 2. Photographs of the heads of chicks inoculated with fowl-pox. Dilution 1:1,000. 5-5, Control chicks on fifth postinoculation day; 5-6, Chicks on a diet containing 1.0% quinine bisulfate; 5-7, Chicks on a diet containing 1.5% quinine bisulfate; 5-8, Chicks on a diet containing 2.0% quinine bisulfate. Left to right, group numbers. Top to bottom, postinoculation days.

pictures of 3 chicks from each group are shown in figure 2 from the fifth to the fifteenth postinoculation days; the lesions healed gradually by the twenty-first to the twenty-eighth day.

In table 2 the average weights for the chicks in the different groups are presented.

TABLE 2

*The effect of quinine upon weight change in chicks inoculated with fowl-pox*

| DIET | AVERAGE WEIGHT OF CHICKS | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Day of inoculation | Days after inoculation | | | | |
| | | | 4 | 7 | 12 | 14 | 21 |
| Inoculum 1:100 | | | | | | | |
| Control | 71 | 77 | 99 | 117 | 152 | 165 | 229 |
| 1.0% | 73 | 67 | 77 | 90 | 106 | 126 | 194 |
| 1.5% | 73 | 65 | 74 | 78 | 94 | 112 | 174 |
| 2 0% | 70 | 58 | 70 | 66 | 84 | 97 | 133 |
| Inoculum 1:1,000 | | | | | | | |
| Control | 71 | 72 | 96 | 106 | 143 | 160 | 206 |
| 1.0% | 73 | 66 | 80 | 87 | 110 | 120 | 179 |
| 1.5% | 73 | 66 | 77 | 90 | 115 | 134 | 190 |
| 2.0% | 70 | 61 | 62 | 66 | 90 | 101 | 150 |

The lack of weight gain in the treated chicks as contrasted with a 50% gain in weight in the control chicks in 4 to 7 days might be considered to be the cause of the marked retardation of development of the lesions in the treated groups, but we feel certain that this is not the case because in our own observations of chicks in other drug-diet mixtures the lesions developed equally as rapidly as in the control chicks, even though the treated chicks lost as much as 25-40% of their original weight in 7 days. Goodpasture (8) also observed that the lesions grew rapidly in chicks which were placed on deficient diets.

## SUMMARY

The effect of quinine in altering the normal course of development of experimental fowl-pox infection in the chick is reported.

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# RELATIVE PHARMACOLOGICAL EFFECTS OF *2*-ALKYL-*1,2,3,4*-TETRAHYDROISOQUINOLINE HYDROCHLORIDES

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The present report is concerned with the relative toxicities and circulatory and smooth muscle effects of three homologous series of *2*-alkyl-*1,2,3,4*-tetrahydroisoquinoline hydrochlorides substituted in the *6*- and *6,7*- positions. The *6,7*-dihydroxy- and *6,7*-dimethoxy- substituted compounds were synthesized by Buck and Ide (1). The chemical aspects of the *6*-ethoxy- substituted compounds are included in the present communication. The pharmacology of the analogous series of unsubstituted *2*-alkyl-*1,2,3,4*-tetrahydroisoquinoline hydrochorides was reported in a previous communication (2), which also gives a bibliography.

EXPERIMENTAL. *I. Chemistry. 2-Alkyl-6-ethoxy-1,2,3,4-tetrahydroisoquinoline hydrochlorides.* *3*-Ethoxyphenethylamine (3) was converted into the formyl derivative which was cyclized by phosphorus oxychloride, in toluene, to give *6*-ethoxy-*3,4*-dihydroisoquinoline (1, 4). This was treated, in benzene solution, with the appropriate alkyl iodide, and the resulting *2*-alkyl-*6*-ethoxy-*3,4*-dihydroisoquinolinium iodide was then reduced with zinc and dilute sulfuric acid. The base was isolated from the reduction mixture and converted into the hydrochloride which was recrystallized from absolute alcohol-ether mixture.

It has previously been reported (5) that *2*-methyl-*6*-ethoxy-*1,2,3,4*-tetrahydroisoquinoline hydrochloride is a powerful depressor. Further examination showed that the specimen contained a second compound formed as a by-product and that the depressor properties were due to this. Consequently, a fresh preparation was made by a method which precluded the formation of the by-product. It is this compound that is at present being reported. The by-product is under investigation.

*II. Toxicity.* In order to obtain the LD 50 values, aqueous solutions were injected intraperitoneally into male albino mice of 15 to 20 gm. body weight. In accordance with previous laboratory procedure, for one week before use the animals were maintained on a diet of equal parts of cracked corn and whole wheat. Prior to injection, they were fasted for 17 hours. Water was allowed *ad lib.* Results were calculated according to the method described by Bliss (6), computations being done graphically. The limits of error given are based upon twice the standard error and give an estimate of the extent to which LD 50 values may be expected to vary because of chance alone. The limits are expressed as per cent and are to be applied to the LD 50 values given in table 2.

In general, toxicity increased as the length of the *2*-alkyl chain increased. This is somewhat emphasized if the LD 50 values are expressed as mM./kgm.

instead of mgm./kgm. The iso derivatives appear to be less toxic than their straight chain isomers. Comparing analogs, the *6,7*-dihydroxy compounds were the least toxic and the *6,7*-dimethoxy compounds the most toxic.

The dose response curves were quite steep, indicating a high degree of uniformity of action. Limits of error are consequently narrow.

Like most compounds with pressor properties, the *6,7*-dihydroxy derivatives produced exophthalmos and pilomotor effects. These symptoms lasted for about an hour when produced by *2*-ethyl-*6,7*-dihydroxy-*1,2,3,4*-tetrahydroisoquinoline hydrochloride. In general, the severity and duration of the symptoms appeared to be associated with the length of the *2*-alkyl chain, the compounds with the longer chains being less effective. Signs of central nervous effects were lacking with the *6,7*-dihydroxy derivatives.

The methoxy and ethoxy derivatives produced coarse tremors but no pilomotor effects, exophthalmos or salivation. The methoxy compounds were also found to be devoid of pressor action, while the ethoxy derivatives were predominantly depressor as noted in table 3.

TABLE 1

*2-Alkyl-6-ethoxy-1,2,3,4-tetrahydroisoquinoline hydrochlorides*

| NO. | ALKYL GROUP | APPEARANCE | M.P. | FORMULA | ANALYSES |  |  |  |
|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | Calcd. |  | Found |  |
|  |  |  |  |  | C | H | C | H |
|  |  |  | °C. (corr.) |  | % | % | % | % |
| 717 | Methyl | Small white flat prisms | 153.5* | $C_{12}H_{18}ONCl$ | 63.27 | 7.97 | 63.55 | 7.97 |
| 718 | Ethyl | Tiny white nodules | 161 | $C_{13}H_{20}ONCl$ | 64.57 | 8.34 | 64.55 | 8.59 |
| 719 | n-Propyl | White powdery platelets | 204 | $C_{14}H_{22}ONCl$ | 65.72 | 8.68 | 65.95 | 8.92 |
| 720 | n-Butyl | White powdery platelets | 214* | $C_{15}H_{24}ONCl$ | 66.76 | 8.97 | 66.92 | 9.25 |

* After drying 12 hrs. in vacuo at 100°C.

The production of coarse tremors by methoxy and ethoxy isoquinoline derivatives has been frequently observed (7) especially in those compounds with analeptic properties.

In addition to the symptoms listed in table 2, all of the above compounds produced short convulsive hops followed by a brief period of coma just before death which appeared to be caused by respiratory failure.

*III. Circulatory effects.* The relative circulatory and respiratory effects are recorded in table 3.

Each compound was tested in two dogs, only one compound being administered per dog. The dogs were anesthetized with dial, 70 mgm./kgm., intraperitoneally. The usual carotid blood pressure tracings were made using a mercury manometer and isotonic sodium citrate as an anticoagulant in the system. The materials were injected into the cannulated femoral vein using physiological saline for flushing.

A standard response to epinephrine was obtained on each dog by administering a dose which would give a pressor response of from 40 to 70 mm. of mercury. This dose was usually 2 micrograms but varied among the dogs from 1 to 4 micrograms per kgm. After standardization to epinephrine, a small dose of the compound under investigation was given. A pulse tracing on a fast drum was obtained. Qualitative respiratory changes were observed and recorded on the chart. After an interval of 10 or more minutes, the standard epinephrine dose was given, in order to observe such adrenolytic, inhibitory or sensitizing effects as the compound might have. At intervals of 20 or more minutes a series of graded doses was given, each dose being double the preceding one. Each dose of the compound was followed by the standard epinephrine dose.

TABLE 2

*Toxicity and symptomatology of 2-alkyl-1,2,3,4-tetrahydroisoquinoline hydrochlorides*

| NO. | SUBSTITUENT GROUPS | MOLECULAR WEIGHT | LD 50 | SLOPE | LIMITS OF ERROR | PILOMOTOR EFFECTS | EXOPHTHALMOS | SALIVATION | TREMORS |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm./kgm.* | | *%* | | | | |
| 778 | *2*-Ethyl-*6*,*7*-dihydroxy | 229.6 | 232 | 20 | 93–108 | Prolonged, marked | Prolonged, marked | Occasional | |
| 779 | *2*-*n*-Propyl-*6*,*7*-dihydroxy | 243.6 | 215 | 38 | 96–105 | Persistent, moderate | Persistent, moderate | Occasional | |
| 780 | *2*-Isopropyl-*6*,*7*-dihydroxy | 243.6 | 243 | 12 | 89–113 | Persistent, slight | Persistent, slight | Occasional | |
| 781 | *2*-*n*-Butyl-*6*,*7*-dihydroxy | 257.6 | 159 | 17 | 89–112 | Brief, slight | Brief, slight | | |
| 782 | *2*-*n*-Amyl-*6*,*7*-dihydroxy | 271.6 | 131 | 14 | 88–113 | Brief, slight | Brief, slight | | |
| 783 | *2*-Isoamyl-*6*,*7*-dihydroxy | 271.6 | 136 | 22 | 91–110 | Occasional, slight | Occasional, slight | | |
| 772 | *2*-Ethyl-*6*,*7*-dimethoxy | 257.6 | 144 | 15 | 89–112 | | | | Coarse |
| 773 | *2*-*n*-Propyl-*6*,*7*-dimethoxy | 271.6 | 177 | 32 | 95–105 | | | | Coarse |
| 774 | *2*-Isopropyl-*6*,*7*-dimethoxy | 271.6 | 248 | 14 | 92–108 | | | | Coarse |
| 775 | *2*-*n*-Butyl-*6*,*7*-dimethoxy | 285.6 | 114 | 22 | 93–107 | | | | Coarse |
| 776 | *2*-*n*-Amyl-*6*,*7*-dimethoxy | 299.7 | 74 | 35 | 96–104 | | | | Coarse |
| 777 | *2*-Isoamyl-*6*,*7*-dimethoxy | 299.7 | 97 | 60 | 96–104 | | | | Coarse |
| 717 | *2*-Methyl-*6*-ethoxy | 227.6 | 106 | 22 | 94–106 | | | | Coarse |
| 718 | *2*-Ethyl-*6*-ethoxy | 241.6 | 150 | 56 | 98–102 | | | | Coarse |
| 719 | *2*-*n*-Propyl-*6*-ethoxy | 255.6 | 187 | 24 | 94–106 | | | | Coarse |
| 720 | *2*-*n*-Butyl-*6*-ethoxy | 269.6 | 146 | 40 | 97–104 | | | | Coarse |

After completion of the graded series, a dose which gave the median response of blood pressure was selected and this was given in order to estimate the possible tachyphylactic effects of the compound under investigation. This "paired" dose was repeated following vagotomy and again following the administration of atropine, 1 mgm./kgm., intravenously, in order to estimate the possible central or peripheral parasympathetic effects.

TABLE 3

*Relative effects of 2-alkyl-1,2,3,4,-tetrahydroisoquinoline hydrochlorides on blood pressure, respiration and pulse*

| NO. | SUBSTITUENT GROUPS | DOSE | | BLOOD PRESSURE | | PAIRED DOSE | EFFECT OF VAGOTOMY | EFFECT OF ATROPINE | EFFECT OF EPINEPHRINE | RESPIRATION | PULSE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Change | Duration | | | | | | |
| | | *ml./kgm.* | *mgm./kgm.* | *mm Hg* | *minutes* | | | | | | |
| 396 | 2-Methyl-6,7-dihydroxy* | .00023 | .05 | p 10 | 1 | | | | S | D | D |
| | | .0175 | 3.77 | p 110 | 15 | | | | | | |
| 778 | 2-Ethyl-6,7-dihydroxy | .001 | .23 | p 28 | 5 | D | 0 | 0 | 0 | D | D |
| | | .008 | 1.79 | p 46 | 30 | | | | | | |
| 779 | 2-n-Propyl-6,7-dihydroxy | .002 | .49 | p 25 | 8 | D | 0 | 0 | 0 | D | D |
| | | .032 | 7.80 | p 43 | 30 | | | | | | |
| 780 | 2-Isopropyl-6,7-dihydroxy | .002 | .49 | p 45 | 8 | 0 | 0 | 0 | 0 | D | D |
| | | .016 | 3.90 | p 67 | 30 | | | | | | |
| 781 | 2-n-Butyl-6,7-dihydroxy | .004 | 1.03 | p 18 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | .032 | 8.24 | p 40 | 8 | | | | | | |
| 782 | 2-n-Amyl-6,7-dihydroxy | 004 | 1.09 | p 48 | 4 | 0 | 0 | 0 | 0 | 0 | D |
| | | 032 | 8.24 | p 77 | 15 | | | | | | |
| 783 | 2-Isoamyl-6,7-dihydroxy | 004 | 1.09 | p 30 | 1 | depressor-pressor | abolished depressor | 0 | D | I | D |
| | | 064 | 17 38 | d 40 p 30 | 1 9 | | | | | | |
| 393 | 2-Methyl-6,7-dimethoxy* | 0029 | 0 71 | d 15 | 6 | | | | R | I | D |
| | | .016 | 3 90 | d 65 | 17 | | | | | | |
| 772 | 2-Ethyl-6,7-dimethoxy | .004 | 1.03 | d 35 | 1 | 0 | 0 | 0 | D | 0 | D |
| | | .032 | 8 24 | d 58 | 3 | | | | | | |
| 773 | 2-n-Propyl-6,7-dimethoxy | .004 | 1 59 | d 40 | 2 | 0 | 0 | 0 | D | I | D |
| | | 016 | 4 35 | d 75 | 10 | | | | | | |
| 774 | 2-Isopropyl-6,7-dimethoxy | .008 | 2.17 | d 25 | 1 | 0 | 0 | 0 | D | 0 | 0 |
| | | .032 | 8 70 | d 45 | 1 | | | | | | |
| 775 | 2-n-Butyl-6,7-dimethoxy | .002 | .57 | d 30 | 1 | 0 | D | 0 | D | D | D |
| | | .016 | 4 57 | d 70 | 3 | | | | | | |
| 775 | 2-n-Amyl-6,7-dimethoxy | .008 | 2.40 | d 17 | 3 | 0 | 0 | 0 | D | 0 | 0 |
| | | .032 | 9.60 | d 40 | 30 | | | | | | |
| 777 | 2-Isoamyl-6,7-dimethoxy | 004 | 1 20 | d 20 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 032 | 9 60 | d 26 | 30 | | | | | | |
| 717 | 2-Methyl-6-ethoxy | 008 | 1.82 | d 25 p 18 | 1 8 | 0 | 0 | 0 | D | I | 0 |
| | | .032 | 7 29 | d 57 p 18 | 2 8 | | | | | | |
| 718 | 2-Ethyl-6-ethoxy | .008 | 1 93 | d 15 p 13 | 1 10 | depressor | 0 | 0 | D | 0 | 0 |
| | | 064 | 15.44 | d 70 | 10 | | | | | | |
| 719 | 2-n-Propyl-6-ethoxy | 008 | 2.05 | d 18 p 10 | 1 3 | depressor | 0 | 0 | D | 0 | 0 |
| | | .064 | 16 36 | d 59 | 2 | | | | | | |
| 720 | 2-n-Butyl-6-ethoxy | .008 | 2.16 | d 7 p 7 | 1 2 | depressor-pressor | 0 | 0 | S | I | D |
| | | 032 | 8 64 | d 48 | 20 | | | | | | |

d, depressor; p, pressor; D, decrease, I, increase; S, sensitized; R, reversed, 0, no change

* These figures are duplicates of those reported elsewhere (5) and (7).

Each compound was tested on two dogs

Only the results of minimum and maximum doses are recorded

*IV. Smooth muscle effects. Isolated organs.* The qualitative effects of these isoquinoline derivatives on the tone of smooth muscle (segments of the small intestine and of the uterus of virgin adult rabbits and the whole uterine horn of virgin adult guinea pigs) are summarized in table 4.

The segments were suspended in Van Dyke-Hastings solution (8) aerated by a mixture of 94% air and 6% carbon dioxide. The tissues were attached to smooth muscle levers and changes of tone were recorded on smoked paper.

*Organs in situ.* The qualitative effects of the 6,7-dihydroxy derivatives on the intact intestine and uterus were tested in 5 non-pregnant cats and one rabbit.

In animals anesthetized with dial, 70 mgm./kgm., intraperitoneally, the changes in intestinal and uterine tone were recorded by the following methods.

TABLE 4

*Effect of 2-alkyl-1,2,3,4-tetrahydroisoquinoline hydrochlorides on tone of isolated smooth muscle*

| NO. | SUBSTITUENT GROUPS | CONCENTRATION IN BATH | RABBIT INTESTINE | RABBIT UTERUS | GUINEA PIG UTERUS | NUMBER OF TESTS |
|---|---|---|---|---|---|---|
| | | *millimolar* | | | | |
| 778 | *2*-Ethyl-*6*,*7*-dihydroxy | 0.2 | – – | + + | + + | 2 |
| 779 | *2*-*n*-Propyl-*6*,*7*-dihydroxy | 0.1 | – – | + + | + + | 2 |
| 780 | *2*-Isopropyl-*6*,*7*-dihydroxy | 0.1 | – – | + + | + + | 2 |
| 781 | *2*-*n*-Butyl-*6*,*7*-dihydroxy | 0.1 | – – | + + | + + | 3 |
| 782 | *2*-*n*-Amyl-*6*,*7*-dihydroxy | 0.1 | – – | + + | + + | 2 |
| 783 | *2*-Isoamyl-*6*,*7*-dihydroxy | 0.1 | – – | + + | + + | 2 |
| 772 | *2*-Ethyl-*6*,*7*-dimethoxy | 0.2 | – – | + + | + + | 2 |
| 773 | *2*-*n*-Propyl-*6*,*7*-dimethoxy | 0.2 | – – | + + | + + | 2 |
| 774 | *2*-Isopropyl-*6*,*7*-dimethoxy | 0.2 | – | + + | + | 2 |
| 775 | *2*-*n*-Butyl-*6*,*7*-dimethoxy | 0.2 | – | + + | + + | 2 |
| 776 | *2*-*n*-Amyl-*6*,*7*-dimethoxy | 0.2 | – | + | + + | 2 |
| 777 | *2*-Isoamyl-*6*,*7*-dimethoxy | 0.1 | – – | + + | + + | 3 |
| 717 | *2*-Methyl-*6*-ethoxy | 0.1 | – | + | + | 2 |
| 718 | *2*-Ethyl-*6*-ethoxy | 0.1 | – – | + + | + + | 3 |
| 719 | *2*-*n*-Propyl-*6*-ethoxy | 0.1 | – – | + + | + + | 3 |
| 720 | *2*-*n*-Butyl-*6*-ethoxy | 0.1 | – – | + + | + + | 3 |

–, moderate decrease; – –, marked decrease; +, moderate increase; + +, marked increase.

A 6-inch loop of intestine was cannulated with a glass tube and ligatured distally, and the intestinal lumen was filled with liquid petrolatum through the glass tube. A large tambour was used for recording changes in volume. The uterus was cut between ligatures at the ovarian end and freed of connective tissues to the base without disturbing the blood supply. A thread tied to the ovarian end was passed over pulleys and attached to a muscle lever. Drugs were injected and blood pressure recorded as described above. The compounds tested were given in 0.1 mM./kgm. doses. Epinephrine was always used as a control.

DISCUSSION. Examination of table 2 reveals that the *6*,*7*-dihydroxy derivatives of the *2*-alkyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline hydrochlorides, with the exception of the *2*-isoamyl compound, were pure pressors with relatively long action. The *6,7*-dimethoxy derivatives of the homologous series were pure depressors. There was little difference in potency but the *2*-amyl and *2*-isoamyl derivatives had the longest depressor action. In small doses the *6*-ethoxy derivatives had a biphasic action consisting of an initial fleeting depressor followed by a longer pressor effect. In large doses they were usually depressor.

It is apparent from the above results that the hydroxy groups in the *6,7* positions conferred pressor potency. This is further evident from the fact that the analogous compounds of the same homologous series having the *6,7* positions unsubstituted were predominantly depressors (2). This corrobates the previous finding that hydroxy groups increase the pressor potency of *1,2,3,4*-tetrahydroisoquinoline hydrochlorides (5), a rule established also for the analogous phenethylamines.

As indicated by the response to paired doses, tachyphylaxis, a well-known property of pressors with ephedrine-like action, was observed only with the long-lasting pressors. Parasympathetic effects were not present among these compounds since vagotomy and atropine had no influence on the blood pressure response to a paired dose. Some evidence for the sympathomimetic action of the pressor compounds was obtained. The responses to paired doses of the *6,7*-dihydroxy-*2*-ethyl, *2*-propyl, and *2*-isopropyl derivatives were increased by cocaine, 10 mgm. per kgm., intravenously, just as the epinephrine response was increased. The reaction to the corresponding dimethoxy derivatives was not increased by cocaine. Although in previous studies (5), cocaine did not increase the pressor action of hydroxy derivatives, other evidence for their sympathomimetic action was deduced.

The pressor response to epinephrine was scarcely influenced by the dihydroxy derivatives but was decreased by the dimethoxy and ethoxy derivatives. As shown previously (7), the *2*-methyl-*6,7*-dimethoxy derivative, in large doses, reversed the epinephrine response. The analogous tetrahydroisoquinoline hydrochlorides having the *6,7* positions unsubstituted (7) were more potent adrenolytics than the present series.

Respiration was not consistently affected by any group of compounds although the dihydroxy derivatives tended to depress while some of the other derivatives stimulated respiration. Pulse was usually slowed by most compounds.

The reactions of isolated smooth muscle to this series of tetrahydroisoquinoline hydrochlorides were remarkably consistent. All of the compounds relaxed the intestine and stimulated both the rabbit and guinea pig uteri. In a previous series of tetrahydroisoquinoline compounds (5), it was observed that hydroxy derivatives usually stimulated the intestine, methoxy derivatives either stimulated or depressed and ethoxy derivatives usually depressed. The direct muscular stimulating action of the tetrahydroisoquinoline hydrochlorides on isolated uteri was observed long ago by Laidlaw (9). The reactions of the isolated intestine, however, point to a direct muscular depressant action on this type of smooth muscle.

The intestine and uterus of the cat and the rabbit in the intact animal were consistently stimulated by each of the *6,7*-dihydroxy derivatives.

## SUMMARY

Three homologous series of *2*-alkyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline hydrochlorides having *6*,*7*-dihydroxy-, *6*,*7*-dimethoxy- and *6*-ethoxy- substituents were studied with respect to toxicity and relative circulatory and smooth muscle effects.

Toxicity increased as the length of the *2*-alkyl chain increased. The *6*,*7*-dihydroxy derivatives were least toxic and the *6*,*7*-dimethoxy group was most toxic. The *6*,*7*-dihydroxy group resembled well-known pressor compounds in the production of pilomotor effects, exophthalmos and salivation. The methoxy and ethoxy groups produced coarse tremors instead of sympathomimetic symptoms.

The hydroxy-substituted compounds were pressors: the shorter carbon chain compounds had the greater potency and duration.

The methoxy-substituted compounds were depressors; the longer carbon chain compounds had the greater potency.

The ethoxy-substituted compounds had biphasic depressor-pressor action with small doses and were usually depressor with large doses.

Tachyphylaxis was observed with some of the pressors.

Vagotomy and atropine had no influence on the blood pressure response.

Respiratory effects were inconsistent.

Pulse rates were usually slowed.

The epinephrine response was not affected by the hydroxy derivatives but was inhibited by methoxy and ethoxy derivatives.

Smooth muscle effects were qualitatively consistent. The isolated intestine was relaxed and the rabbit and guinea pig uteri were stimulated by all the compounds. The *6*,*7*-dihydroxy derivatives stimulated the intestine and uterus of the intact cat and rabbit.

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# RELATIVE PHARMACOLOGICAL EFFECTS OF *2,2*-DIMETHYL-*1,2,3,4*-TETRAHYDROISOQUINOLINIUM CHLORIDES

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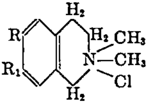
This report deals with the chemical and pharmacological properties of a series of quaternary tetrahydroisoquinolinium chlorides, having hydroxy, methoxy, and ethoxy groups in the *6* and *6,7* positions. The general formula is as follows:



where $R$ and $R_1$ are hydroxy, methoxy or ethoxy groups, or hydrogen. The secondary and tertiary analogs have been studied previously (1).

EXPERIMENTAL. *I. Chemistry.* For the present work, the *2*-methyl-alkoxy-*1,2,3,4*-tetrahydroisoquinoline compounds and the dialkoxy analogs were all obtained via the methiodides of the corresponding *3,4*-dihydroisoquinoline compounds, which were reduced with zinc and sulfuric acid.

The *2*-methyl-alkoxy-*1,2,3,4*-tetrahydroisoquinoline bases and the dialkoxy analogs were readily converted into the corresponding methiodides by the action of methyl iodide in benzene solution. The methiodide in aqueous solution, on treatment with silver chloride, gave the corresponding quaternary chloride. Heating with conc. hydrochloric acid at 160-170°C. demethylated the chlorides of the methoxy and dimethoxy compounds giving the hydroxy and dihydroxy compounds in good yield.

*II. Toxicity.* The methods used for the determination of LD 50 values were described in a previous paper (2). The results are given in table 2. Comparatively speaking, these compounds were quite toxic. This is consistent with other data on quaternary tetrahydroisoquinolinium salts (3). The lack of more pronounced sympathomimetic symptoms is noteworthy in view of the pressor actions of these compounds. In all cases, a few short convulsive hops followed by a brief period of coma preceded death, which appeared to be due to respiratory failure.

*III. Circulatory effects.* The general circulatory effects are summarized in table 3. The methods used were identical with those of a previous report (2).

This representative series of quaternary tetrahydroisoquinolinium chlorides, with one exception, were pure pressors. The *6*-hydroxy derivative had an initial fleeting depressor effect followed by a long pressor action. The analogous secondary and tertiary methoxy and ethoxy tetrahydroisoquinoline compounds

TABLE 1

*2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium chlorides*

| NO. | SUBSTITUENT GROUPS | APPEARANCE | M.P. | FORMULA | ANALYSES Calcd. C | Calcd. H | Found C | Found H | SOLVENT RECRY-ST. |
|---|---|---|---|---|---|---|---|---|---|
| | | | °C. (corr.) | | % | % | % | % | |
| 725 | 6-Hydroxy | Grayish flat pointed prisms | 275.5 dec. | $C_{11}H_{16}ONCl$ | 61.80 | 7.55 | 61.96 | 7.62 | M E |
| 726 | 6,7-Dihydroxy | Pale fawn glittering flat leafy prisms | Above 285 | $C_{11}H_{16}O_2NCl$ | 57.49 | 7.03 | 57.36 | 7.15 | aA E |
| 721 | 6-Methoxy | Bulky tiny glittering meshed prisms | 176* | $C_{12}H_{18}ONCl$ | 63.27 | 7.97 | 63.35 | 8.04 | Bu E |
| 723 | 6-Ethoxy | Beautiful white silky leaves | 162.5* | $C_{13}H_{20}ONCl$ | 64.57 | 8.34 | 64.79 | 8.49 | A E |
| 722 | 6,7-Dimethoxy | Tiny thin glittering plates | 267–268 dec. | $C_{13}H_{20}O_2NCl$ | 60.56 | 7.83 | 60.42 | 8.09 | M E |
| 724 | 6,7-Diethoxy | Glittering small plates | 226.5* | $C_{15}H_{24}O_2NCl$ | 63.02 | 8.47 | 63.03 | 8.55 | A E |

M, methanol; A, ethanol; a, aqueous; Bu, butanol; E, ether.

* Dried in vacuo for 12 hrs. at 100°C.

725 By demethylation of 721.

726 By demethylation of 722.

721 Cf. Buck, J. S.: J. Am. Chem. Soc., 54: 3661, 1932; Helfer: Helv. Chim. Acta, 7: 945, 1924 for intermediate.

723 Cf. Ide, W. S., and Buck, J. S.: J. Am. Chem. Soc., 59: 726, 1937 for intermediate.

722 From 2-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (base). Cf. Pyman: J. Chem. Soc. (Lond.), 95: 1266, 1909; Buck, J. S.: J. Am. Chem. Soc., 56: 1769, 1934.

724 Cf. Ide, W. S., and Buck, J. S.: loc. cit., for intermediate.

TABLE 2

*Toxicity and symptomatology of 2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium chlorides*

| NO. | SUBSTITUENT GROUPS | MOLECULAR WEIGHT | LD 50 | SLOPE | LIMITS OF ERROR | PILOMOTOR EFFECTS | EXOPHTHALMOS | SALIVATION | TREMORS |
|---|---|---|---|---|---|---|---|---|---|
| | | | mgm./kgm. | | % | | | | |
| 725 | 6-Hydroxy | 213.6 | 25 | 35 | 97–103 | In large doses | In large doses | | Coarse |
| 726 | 6,7-Dihydroxy | 229.6 | 33 | 20 | 91–110 | In large doses | In large doses | | |
| 721 | 6-Methoxy | 227.6 | 31 | 9 | 85–118 | | | | Coarse |
| 723 | 6-Ethoxy | 241.6 | 58 | 22 | 93–108 | | | | |
| 722 | 6,7-Dimethoxy | 257.6 | 20 | 18 | 93–108 | Brief, marked | Brief, marked | | |
| 724 | 6,7-Diethoxy | 285.7 | 69 | 6 | 84–120 | | | | Coarse |

### TABLE 3

*Relative effects of 2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium chlorides on blood pressure, respiration and pulse*

| NO. | SUBSTITUENT GROUPS | DOSE | | BLOOD PRESSURE | | PAIRED DOSE | EFFECT OF VAGOTOMY | EFFECT OF ATROPINE | EFFECT ON EPINEPHRINE | RESPIRATION | PULSE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Change | Duration | | | | | | |
| | | *mM./kgm.* | *mgm./kgm.* | *mm. Hg* | *minutes* | | | | | | |
| 725 | *6*-Hydroxy | 004<br>.032 | .86<br>6.84 | d 40 p 19<br>d 40 p 90 | <1 5<br><1 15 | 0 | Abolished depressor | 0 | S | I | D |
| 726 | *6,7*-Dihydroxy | .001<br>.008 | .23<br>1.84 | p 35<br>p 150 | 2<br>15 | 0 | 0 | 0 | S | I | D |
| 721 | *6*-Methoxy | .0005<br>.004 | .113<br>.91 | p 11<br>p 152 | 1<br>5 | 0 | 0 | D | S | I | D |
| 723 | *6*-Ethoxy | .002<br>.032 | .48<br>7.72 | p 25<br>p 110 | 2<br>20 | 0 | 0 | D | S | I | D |
| 722 | *6,7*-Dimethoxy | .00025<br>.004 | .064<br>1.03 | p 10<br>p 150 | 1<br>6 | 0 | 0 | D | S | I | D |
| 724 | *6,7*-Diethoxy | .002<br>.008 | .57<br>2.28 | p 46<br>p 110 | 3<br>6 | 0 | 0 | 0 | S | I | D |

D, Decrease; I, increase; S, sensitized; 0, no change or effect; d, depressor; p, pressor. Each compound was tested on two dogs.

### TABLE 4

*Effect of 2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium chlorides on tone of isolated smooth muscle*

| NO. | SUBSTITUENT GROUPS | CONCENTRATION IN BATH | RABBIT INTESTINE | RABBIT UTERUS | GUINEA PIG UTERUS | NUMBER OF TESTS |
|---|---|---|---|---|---|---|
| | | *milli-molar* | | | | |
| 725 | *6*-Hydroxy | 0.1-0.2 | (+ —) | + + | + | 2 |
| 726 | *6,7*-Dihydroxy | 0.1-0.2 | (+ —) | + + | + + | 2 |
| 721 | *6*-Methoxy | 0.1-0.2 | (+ —) | + + | + + | 5 |
| 723 | *6*-Ethoxy | 0 1-0.2 | — — | + + | + + | 2 |
| 722 | *6,7*-Dimethoxy | 0.1-0 2 | — | + | + + | 2 |
| 724 | *6,7*-Diethoxy | 0.1-0.2 | — — | + + | + + | 2 |

—, moderate decrease; — —, marked decrease; +, moderate increase; + +, marked increase; (+ —), increase or decrease.

were depressors or diphasic depressor-pressors (1). Apparently the conversion of the secondary and tertiary amines to quaternary salts has conferred pressor potency on the isoquinoline nucleus.

There was no evidence of tachyphylaxis among these pressors since paired doses gave equivalent responses. Vagotomy abolished the depressor phase of the *6*-hydroxy derivative, indicating that this was a central vagus stimulating action. Vagotomy had no effect on the activity of the other compounds. Atropine inhibited the paired dose response of three of the compounds. A similar inhibitory action of atropine on the pressor effect has been found for quaternary salts of phenethylamine derivatives (unpublished data.). The epinephrine response was potentiated by each compound. Respiration was consistently stimulated during the early part of the blood pressure action. Pulse was consistently slowed.

*IV. Smooth muscle effects.* The smooth muscle effects, summarized in table 4, were obtained on isolated tissues by methods previously described (2).

The first three compounds had mixed effects on the intestine, at times either stimulating or relaxing, while the other compounds consistently relaxed the intestine. All of the compounds stimulated both the rabbit and guinea pig uteri, a reaction characteristic of many isoquinoline derivatives (2).

## SUMMARY

Representative quaternary tetrahydroisoquinolinium chlorides, substituted in the *6* and *6,7* positions with hydroxy, methoxy and ethoxy groups, were studied for their relative toxicities and effects on circulation and smooth muscle.

In accordance with other data on quaternary tetrahydroisoquinolinium chlorides these compounds have been found to be relatively quite toxic.

These quaternary tetrahydroisoquinolinium chlorides were predominantly pressor in contrast to analogous secondary and tertiary isoquinoline derivatives.

Vagotomy abolished the depressor phase of the *6*-hydroxy derivative, and atropine inhibited the pressor phase of three of the compounds.

Respiration was consistently stimulated.

Pulse was consistently slowed.

Epinephrine was sensitized by all the compounds.

Isolated intestine was depressed by some compounds and both stimulated and depressed by others. Rabbit and guinea pig uteri were stimulated.

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# STUDIES ON THE TOXICITY AND PHARMACOLOGY OF RIBOFLAVIN

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Nutritional research during the last decade has shown that riboflavin, *6,7*-dimethyl-*9*-[*1′*-*d*-ribityl]- isoalloxazine, is essential for the growth of bacteria and for the nutrition of a wide variety of animal species including chickens, rats, dogs and swine. Its importance in the nutrition of man was demonstrated by Sebrell and Butler (1) who by experimental deprivation of riboflavin produced a deficiency disease, "ariboflavinosis." Their observations and the response of this deficiency syndrome to the administration of riboflavin have been confirmed by numerous clinicians and it has been stated recently (2) that ariboflavinosis is possibly the most prevalent uncomplicated avitaminosis. In view of the widespread recognition of the importance of riboflavin and of the recommendation to restore to flour and bread the riboflavin which is lost through the milling process, it has seemed advisable to study its toxicologic and pharmacologic effects.

Kuhn and Boulanger (3) failed to produce toxic effects in a mouse following the intraperitoneal injection of 340 mgm. of sodium riboflavinate per kgm. body weight. Demole (4) found riboflavin to be non-toxic to fishes, frogs, mice, rabbits, cats and dogs following single or repeated doses ranging from 1 to 50 mgm. per kgm. body weight. Rats tolerated the oral administration of 40 mgm. of riboflavin repeated on 4 consecutive days. Widenbauer and Wedeweyer (5), upon feeding riboflavin in doses varying between 0.15 and 1 mgm. to a small group of rats over periods extending to 36 days, likewise, failed to observe toxic effects.

Crystalline riboflavin is soluble in water only to an amount of 11 mgm. in 100 cc. Sodium riboflavinate possessing the same vitamin activity as the riboflavin base is more soluble in water (2%). However, the large doses required in the toxicity studies could be administered only in the form of 2 to 10% aqueous suspensions of riboflavin or of sodium riboflavinate.

ACUTE TOXICITY. Oral administration of aqueous suspensions of 10 grams of riboflavin or sodium riboflavinate per kgm. body weight was tolerated by rats without evidence of toxic effects. Likewise, the subcutaneous injection of riboflavin in doses of 5 grams per kgm. produced no toxic effects. Failure to absorb riboflavin in sufficient quantities under these conditions was suggested by the intensive yellow color of the faeces of the rats and the findings of massive deposits of riboflavin at the site of the subcutaneous injections as long as 10 days after the administration. Sodium riboflavinate, however, was more readily absorbed from the subcutaneous tissues. From the peritoneal cavity, riboflavin and sodium riboflavinate seemed to be equally well absorbed. Only traces of the vitamin were found in the peritoneal cavity of rats 3 days after the intraperitoneal injection of 300 mgm. per kgm., and no difference in toxicity between the two compounds was observed when this mode of administration was employed. The results obtained from the total of 400 rats and expressed by the L.D. 50 following oral, subcutaneous and intraperitoneal administration are summarized in table 1. The low solubility of the vitamin precluded a determination of the toxicity following intravenous injection.

The injection of toxic doses was followed by a period of anuria for 1 or 2 days, and only scant amounts of deep yellow colored urine were excreted after this period. The rats became listless, refused food, and in spite of the anuria, lost rapidly in weight. Tremors were frequently observed. Death occurred within 2 to 5 days. On autopsy, the kidneys had a mottled brownish yellow appearance and were found to contain considerable amounts of bright yellow crystals in the collecting tubules and the pelvis, as shown in figure 1. Concretions were found in the kidneys of all animals killed either by riboflavin following intraperitoneal administration or by sodium riboflavinate following intraperitoneal and subcutaneous injection. The histopathologic findings on the kidneys will be described elsewhere by Dr. Wm. Antopol (6). Through the courtesy of Dr. J. V. Scudi, determinations of urea by Karr's method (7), and of creatinine

TABLE 1

*Acute toxicity of riboflavin and sodium riboflavinate in rats (150–200 grams)*

L.D. 50 (gram per kilogram body weight)

| | ORAL | SUBCUTANEOUS | INTRA-PERITONEAL |
|---|---|---|---|
| Riboflavin | >10.0 | 5.0 | 0.56 |
| Sodium riboflavinate | >10.0 | 0.79 | 0.56 |

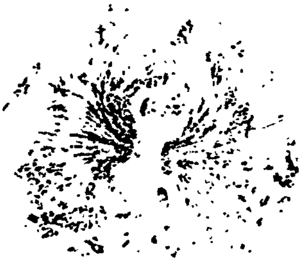

FIG. 1. CONCRETIONS (BRIGHT YELLOW CRYSTALS) IN THE KIDNEY OF A RAT 48 HOURS AFTER INTRAPERITONEAL INJECTION OF 600 MGM. OF RIBOFLAVIN PER KGM. BODY WEIGHT

by the method of Folin-Wu (8), were made in the blood of 8 rats injected with 600 mgm. of riboflavin per kgm. The determinations carried out after a 12 hour fasting period on the second day following the administration of riboflavin revealed a ten-fold increase in urea and a three to five-fold increase in the creatinine, whereas the blood levels in a control group of 6 rats fell within the normal range.

In an attempt to accelerate the excretion of riboflavin in order to prevent or modify the formation of crystals in the kidney, 3 groups of rats were given water in an amount of 1, 2½ or 5% of body weight respectively, 4 times every 24 hours by stomach tube. In rats receiving large amounts of water, the period of anuria was shortened to 12 hours. The forced feeding of water, however, did not prevent the formation of concretions and no difference in survival time was found between the groups receiving different amounts of water.

Following oral or subcutaneous administration of riboflavin in doses of 10 and 5 grams per kilogram respectively, no crystals were found in the kidneys.

A peroral dose of 2 grams of riboflavin per kilogram was tolerated by 3 dogs without evidence of toxic symptoms. There was no change in the amount of urine excreted. Microbiological determinations of the riboflavin content of the urine, kindly performed by Dr. R. H. Silber, revealed that only very small fractions of the dose ingested were excreted in the urine: 0.1% of the dose of 2 grams per kilogram were recovered during the first 24 hours. Thereafter the excretion of riboflavin fell off rapidly and returned to normal levels after 12 days. The dogs were sacrificed 8 hours, 36 hours and 17 days respectively after the administration of riboflavin. Microscopic examination of the kidneys failed to show pathologic changes.

Chronic toxicity. Daily doses of 10 mgm. of riboflavin were fed to weanling male and female rats over a period of 140 days. . The animals showed normal development and their average growth rate did not differ significantly from that of a control group (table 2). The animals were mated, and normal litters averaging 10 young rats were obtained from both the riboflavin fed group and the control group. Offspring of the first generaion were again fed daily with 10 mgm. riboflavin after they reached the age of 3 weeks. Daily feeding of riboflavin over periods of 140 days were continued through 3 generations. No difference in the development, growth, maturation or reproduction was observed between the rats fed riboflavin and those of the control group (table 2). Premature birth of offspring, claimed to be caused by the administration of riboflavin in pregnant rats (9), did not occur in these experiments. Autopsies at the end of the feeding period did not reveal any gross changes in the organs.

Four young dogs, started at 10 weeks of age, were fed 25 mgm. per kilogram of riboflavin daily over a period of 5 months, with 2 litter-mates serving as controls. Their growth was normal and no toxic symptoms were observed. All dogs were sacrificed at the end of the feeding period. Macroscopic examination of the organs failed to reveal any changes.

Effect on metabolism. The action of riboflavin on the metabolism of rats was studied by the method of Richards and Collison (10). The rats were deprived of food for 16 hours prior to the oral administration of 1 or 10 mgm. of riboflavin respectively. No change in oxygen consumption was found in these rats on observing the animals for 3 hours after the ingestion of the vitamin. Furthermore, the oxygen consumption of rats fed 10 mgm. of riboflavin daily was found unchanged after a period of 9 days.

In view of the anuria observed following lethal doses of riboflavin, the effect of orally administered riboflavin on water metabolism was studied in 16 rats of 300 gram average body weight. The diuresis of groups of 4 rats was followed using the diuresis recorder of Kniazuk (11). The normal excretion following 5 cc. of tap water per 100 gram body weight was first established for each group. Doses of 1 or 10 mgm. of riboflavin respectively given simultaneously with the water changed neither the rate nor the amount of urine excreted (fig. 2).

TABLE 2

*Growth of young rats receiving riboflavin daily by mouth*

| | AVERAGE WEIGHT (GRAMS) AFTER | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 days | | 50 days | | 100 days | | 140 days | |
| | Males | Females | Males | Females | Males | Females | Males | Females |
| Control. Stock diet only: | | | | | | | | |
| 11 males, 11 females, 1st generation | 96 | 87 | 222 | 156 | 294 | 207 | 317 | 237 |
| 12 males, 12 females, 2nd generation | 83 | 78 | 123 | 156 | 349 | 225 | 405 | 242 |
| 12 males, 12 females, 3rd generation | 91 | 80 | 241 | 169 | 339 | 214 | 381 | 239 |
| 10 mgm. riboflavin daily: | | | | | | | | |
| 10 males, 10 females, 1st generation | 98 | 83 | 230 | 154 | 317 | 206 | 347 | 243 |
| 12 males, 10 females, 2nd generation | 95 | 88 | 235 | 167 | 344 | 236 | 412 | 259 |
| 12 males, 12 females, 3rd generation | 91 | 84 | 239 | 171 | 351 | 219 | 416 | 246 |

EFFECT ON CIRCULATION AND SMOOTH MUSCLES. The blood pressure and the respiration of 6 cats under nembutal or dial anesthesia were not significantly influenced by intravenous injections of sodium riboflavinate in doses ranging from 2 to 40 mgm. per kgm. The heart rate recorded simultaneously with the blood pressure by the method of Unna and Kniazuk (12) remained unchanged.

On the smooth muscles of the isolated intestine of the rabbit, the vitamin was likewise without effect. Concentrations of sodium riboflavinate up to 1:10,000 did not alter the intestinal tone nor the rythmic contraction. Spasms of the intestine induced by acetyl choline were not influenced by the vitamin.

DISCUSSION. Riboflavin administered in excessive amounts by mouth was found to be non-toxic in dogs and rats. In this respect it resembles pantothenic acid (13), but differs from other vitamins of the B complex, thiamin (14), nicotinamide (15), or pyridoxine (16), (table 3). The absence of toxic effects may be explained by a failure to absorb the vitamin in sufficient quantities from the gastro-intestinal tract. Following intraperitoneal injection, however, riboflavin produced death due to kidney concretions and ensuing anuria and azotemia, its toxicity being of the same order of magnitude as that of pyridoxine or pantothenic acid. It is the only member of the group of B vitamins other than pyridoxine (16) which may produce specific toxic manifestations when administered in excessive doses.

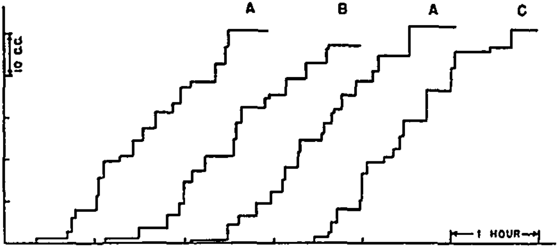

FIG. 2. CONTINUOUS RECORDING OF THE DIURESIS OF 4 RATS FOLLOWING A DOSE OF 5 cc. OF TAP WATER PER 100 GRAMS BODY WEIGHT

A, water alone; B, water and 1 mgm. riboflavin per rat; C, water and 10 mgm. riboflavin per rat.

TABLE 3

*Comparison of the L.D. 50 of riboflavin, nicotinamide, pyridoxine and pantothenic acid in rats following oral, subcutaneous and intravenous administration*

| | ORAL | SUBCUTANEOUS | INTRAVENOUS |
|---|---|---|---|
| Riboflavin | >10.0 | >5.0 | 0.560* |
| Nicotinamide | 3.5 | 2.7 | 2.2 |
| Pyridoxine | 5.5 | 3.7 | 0.657† |
| Pantothenic acid | >10.0 | 3.4 | 0.830 |

* Intraperitoneal administration.

† According to Weigand, Eckler and Chen, Proc. Soc. Exp. Biol. & Med., **44**: 147, 1940.

The absence of any toxic effects in aimals fed riboflavin in amounts representing about a thousand times the daily requirement in experiments extending over periods of 5 months corresponds well with similar findings on pyridoxine and pantothenic acid. Furthermore, the pharmacological inertness of riboflavin in animals maintained on adequate diets parallels that of previously studied members of the vitamin B complex.

*Acknowledgement.* Appreciation is expressed to Dr. H. Siegel for the pathological studies and to Mrs. T. M. Edison for the measurement of the oxygen consumption reported in this paper.

## SUMMARY

1. The L.D. 50 of riboflavin in rats following intraperitoneal administration is 560 mgm. per kgm. Death occurs within 2 to 5 days with signs of anuria and azotemia and is due to obstruction of the kidney by concretions.

2. Oral administration of riboflavin to rats (10 grams per kgm.) and to dogs (2 grams per kgm.) failed to produce any toxic effects. The low solubility of riboflavin prevents its absorbtion from the gastro intestinal tract in amounts sufficient to produce toxic effects.

3. Daily administration of riboflavin over periods of 4 months to rats (10 mgm.) and dogs (25 mgm. per kgm.) failed to produce any toxic manifestations. Rats receiving 10 mgm. daily were raised through 3 generations.

4. The metabolism, the circulatory and respiratory systems, and isolated smooth muscle organs of animals maintained on adequate diets are not influenced by riboflavin.

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# SYNTHETIC DERIVATIVES OF STROPHANTHIDIN[1]

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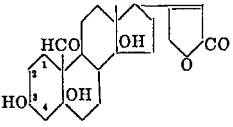
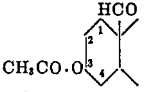
In a previous investigation (2), several derivatives of cymarin and strophanthidin were studied with the purpose of determining the relative importance of chemical groupings in the two molecules. The results are instructive and add to our understanding of digitalis chemistry in general. Eventually it is hoped that synthetic products having a digitalis-like action may be developed. Since the cardiac action depends among other factors upon the presence of the unsaturated lactone ring, a series of simple lactones were prepared (3–11) and subjected to pharmacologic investigation (12). Indeed, β,γ-angelica lactone, and methyl and ethyl coumalates showed some evidence of a digitalis-like action in frogs, but not in cats. Krayer, Mendez, and de Espanés (13) observed similar effects on the isolated frog's heart with β,γ-angelicalactone, α,β-angelicalactone, crotonolactone γ-acetic acid, and the methyl ester of crotonolactone γ-acetic acid. Linstead and Krayer (14) also demonstrated the systolic effect of *l*-ascorbic acid, which has a lactone ring, on the frog's heart.

Another line of approach has been to utilize the molecules of aglycones, such as strophanthidin, and to introduce various radicles on to the steroid ring system through the hydroxyl groups. Seven synthetic glycosides and the acetate of strophanthidin have now been prepared in the department of chemistry, Columbia University. The chemical procedures will be presented elsewhere (15). The present communication deals with the comparison of potencies of these substances in cats and frogs.

The names of the compounds and their partial structural formulas are shown as follows:



Strophanthidin



Strophanthidin Acetate

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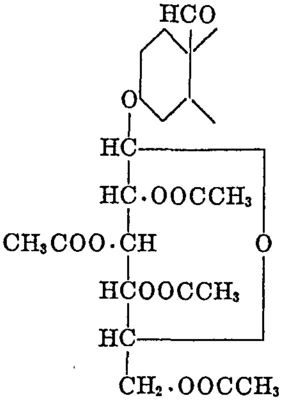
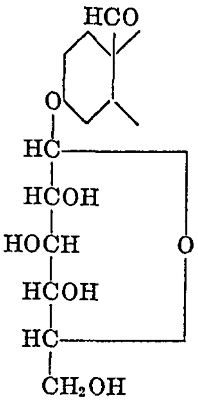
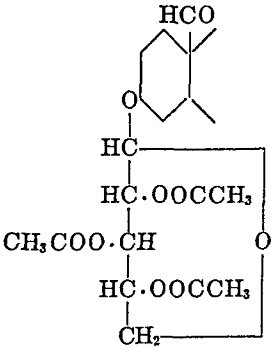
[1] Read in part at the Boston meeting of the Federation of American Societies for Experimental Biology, 1942 (1)



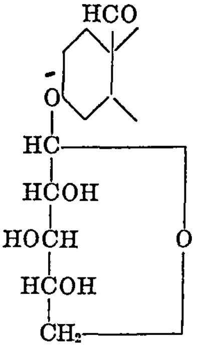
Strophanthidin-β-tetraacetyl-*d*-glucoside



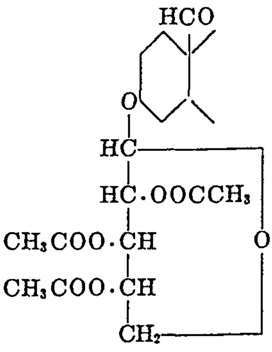
Strophanthidin-β-*d*-glucoside



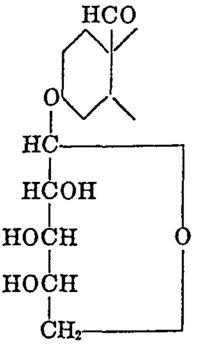
Strophanthidin-β-triacetyl-*d*-xyloside



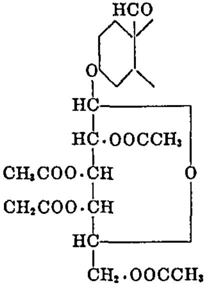
Strophanthidin-β-*d*-xyloside



Strophanthidin-β-triacetyl-*l*-arabinoside



Strophanthidin-β-*l*-arabinoside



Strophanthidin-β-tetraacetyl-*d*-galactoside

It is obvious that in each case substitution takes place at the secondary OH-group on carbon atom 3 of strophanthidin. The acetate has been previously prepared and investigated by Neumann (16).

For animal experiments, a stock solution of 0.1 per cent of each compound was made. Since the glycosides are not entirely soluble in water, various amounts of ethyl alcohol were required—33.25% by volume for strophanthidin-β-tetraacetyl-*d*-glucoside, -triacetyl-*l*-arabinoside, and -*d*-xyloside, and strophanthidin acetate; 47.5% for strophanthidin-β-*l*-arabinoside and -*d*-glucoside; 57% for strophanthidin-β-tetraacetyl-*d*-galactoside; and 95% for strophanthidin-β-triacetyl-*d*-xyloside. Dilutions of 1:100,000 of strophanthidin-β-*l*-arabinoside, -*d*-xyloside, and -*d*-glucoside, and strophanthidin acetate were employed for intravenous injection in cats. Dilutions of 1:12,500 of strophanthidin-β-tetraacetyl-*d*-glucoside, -tetraacetyl-*d*-galactoside, and -triacetyl-*l*-arabinoside, were used for the same purpose. Strophanthidin-β-triacetyl-*d*-xyloside is so insoluble that a 0.1% solution in 95% alcohol by volume was injected at the rate of 0.04 cc. per minute, each time followed by 1 cc. of saline solution—in the same manner as in the case of scillaridin A (17).

In frogs strophanthidin-β-*l*-arabinoside, -*d*-xyloside, and -*d*-glucoside were administered in 1:20,000 concentrations; strophanthidin acetate in 1:4000; and the remaining members of the series in 1:1000.

Preliminary tests indicated that each of the above compounds possesses a digitalis-like action as evidenced by systolic standstill of the frog's ventricle and emesis in cats following sublethal doses given by vein. During the slow injection of dilute solutions intravenously, diminution of the heart rate, premature beats, arrhythmia, secondary tachycardia, and finally ventricular fibrillation, could be distinguished by auscultation in etherized cats. Their action upon the heart resembling that of digitalis was unmistakable. This is to be expected because the lactone ring and the stereochemical isomerism have remained intact in all the molecules.

TABLE 1

*Assay in cats*

| COMPOUND | SEX | BODY WEIGHT | DOSE TO KILL | MEAN LETHAL DOSE ± STANDARD ERROR |
|---|---|---|---|---|
| | | *kgm.* | *μgm. per kgm.* | *μgm. per kgm.* |
| Strophanthidin | F | 2.632 | 403.3 | 306.2 ± 38.7 |
| | F | 1.537 | 227.7 | |
| | F | 2.608 | 372.7 | |
| | M | 1.930 | 223.8 | |
| | F | 2.346 | 351.2 | |
| Strophanthidin acetate | F | 2.844 | 123.1 | 186.6 ± 24.6 |
| | M | 1.772 | 272.6 | |
| | F | 1.956 | 191.2 | |
| | M | 1.633 | 212.5 | |
| | F | 1.699 | 166.0 | |
| Strophanthidin-β-tetra-acetyl-*d*-glucoside | F | 2.065 | 1313.3 | 1166 ± 125 |
| | M | 2.058 | 1648.2 | |
| | F | 2.417 | 926.4 | |
| | F | 2.174 | 953.1 | |
| | F | 1.944 | 1130.0 | |
| Strophanthidin-β-*d*-glucoside | M | 2.198 | 98.36 | 91.3 ± 2.46 |
| | M | 2.663 | 86.11 | |
| | F | 2.525 | 91.01 | |
| | F | 2.216 | 85.42 | |
| | F | 1.740 | 98.85 | |
| | F | 1.854 | 80.91 | |
| | M | 1.969 | 86.08 | |
| | F | 2.246 | 106.18 | |
| | F | 1.772 | 95.93 | |
| | F | 1.965 | 87.02 | |
| Strophanthidin-β-triacetyl-*d*-xyloside | F | 2.210 | 579.2 | 591.6 ± 70.4 |
| | M | 1.873 | 662.0 | |
| | F | 2.424 | 379.5 | |
| | F | 2.077 | 654.8 | |
| | F | 2.263 | 760.1 | |
| Strophanthidin-β-*d*-xyloside | F | 2.765 | 102.71 | 109.5 ± 4.39 |
| | F | 1.852 | 93.41 | |
| | F | 2.226 | 104.67 | |
| | M | 1.862 | 96.13 | |
| | F | 1.808 | 109.51 | |
| | F | 1.714 | 111.43 | |
| | M | 2.301 | 144.93 | |
| | F | 1.655 | 103.92 | |
| | M | 1.718 | 122.81 | |
| | F | 1.968 | 113.82 | |

TABLE 1—*Concluded*

| COMPOUND | SEX | BODY WEIGHT | DOSE TO KILL | MEAN LETHAL DOSE ± STANDARD ERROR |
|---|---|---|---|---|
| | | *kgm.* | *µgm. per kgm.* | *µgm. per kgm.* |
| Strophanthidin-β-triacetyl-*l*-arabinoside | F | 2.435 | 1278.0 | 1230 ± 136.6 |
| | F | 1.802 | 1784.7 | |
| | M | 2.096 | 954.2 | |
| | M | 1.843 | 1284.9 | |
| | F | 1.645 | 1006.7 | |
| Strophanthidin-β-*l*-arabinoside | F | 2.348 | 81.77 | 94.5 ± 2.95 |
| | F | 2.370 | 90.08 | |
| | F | 2.289 | 107.91 | |
| | F | 1.909 | 99.00 | |
| | M | 2.117 | 88.80 | |
| | M | 1.659 | 88.00 | |
| | F | 1.884 | 113.32 | |
| | F | 1.979 | 91.46 | |
| | F | 1.877 | 91.10 | |
| | F | 1.663 | 97.41 | |
| Strophanthidin-β-tetraacetyl-*d*-galactoside | F | 1.862 | 1695.0 | 1692 ± 168 |
| | F | 1.933 | 1536.5 | |
| | M | 2 358 | 1911.8 | |
| | F | 1.892 | 2241.0 | |
| | F | 2 195 | 1246.5 | |
| Cymarin | | | | 110.1 ± 3.75* |

* This figure is the mean (geometric) of combined data published previously (18, 19).

It was thus decided to study these substances in a sufficient number of cats and frogs, so that their potencies could be precisely determined. The method for cats was the same as that previously described (18), and that for frogs, according to the U.S.P.XI (20). The results show a similar trend, and are summarized in tables 1 and 2. It should be noted that strophanthidin acetate is more potent than strophanthidin, confirmatory of Neumann's findings (16). The introduction of an acetyl group to the steroid ring system, however, may produce an opposite effect. Previously, it was demonstrated that acetylation of cinobufagin and marino-bufagin reduced the cardiac activity (21). On the other hand, deacetyl-oleandrin was shown to be less potent than oleandrin (22, 23).

The four acetyl-glycosides are much less active than strophanthidin. The presence of a completely acetylated sugar molecule, therefore, exerts an unfavorable influence upon the activity of the aglycone. Incidentally, low solubility in water is apparently not always attended by poor absorption and a relatively lower potency. For example, strophanthidin-β-triacetyl-*d*-xyloside, being only soluble in 95% alcohol, is stronger in both cats and frogs than the more soluble compounds, strophanthidin-β-tetraacetyl-*d*-glucoside and -tetraacetyl-*d*-galactoside.

TABLE 2

*Assay in frogs*

| COMPOUND | DOSE | NUMBER IN SYSTOLE/ NUMBER USED | $SD_{50} \pm$ S.E. |
|---|---|---|---|
| | *μgm. per gm.* | | *μgm. per gm.* |
| Strophanthidin | 1.6 | 0/5 | 2.71 ± 0.49 |
| | 2.0 | 2/5 | |
| | 2.5 | 2/5 | |
| | 3.0 | 4/5 | |
| | 5.0 | 2/2 | |
| Strophanthidin acetate | 1.0 | 0/5 | 2.19 ± 0.13 |
| | 1.6 | 1/10 | |
| | 2.0 | 3/10 | |
| | 2.5 | 7/10 | |
| | 3.0 | 9/10 | |
| Strophanthidin-β-tetraacetyl-*d*-glucoside | 5.0 | 0/2 | 18.77 ± 3.07 |
| | 10.0 | 2/5 | |
| | 14.0 | 2/5 | |
| | 20.0 | 1/5 | |
| | 25.0 | 2/5 | |
| | 30.0 | 5/5 | |
| Strophanthidin-β-*d*-glucoside | 0.35 | 1/10 | 0.583 ± 0.04 |
| | 0.40 | 2/10 | |
| | 0.50 | 3/15 | |
| | 0.60 | 6/10 | |
| | 0.70 | 8/10 | |
| | 0.90 | 4/5 | |
| Strophanthidin-β-triacetyl-*d*-xyloside | 7.0 | 1/5 | 8.07 ± 1.35 |
| | 10.0 | 3/5 | |
| | 14.0 | 4/5 | |
| | 20.0 | 5/5 | |
| Strophanthidin-β-*d*-xyloside | 0.40 | 1/5 | 0.64 ± 0.04 |
| | 0.50 | 1/10 | |
| | 0.60 | 3/10 | |
| | 0.70 | 8/10 | |
| | 0.90 | 4/5 | |
| Strophanthidin-β-triacetyl-*l*-arabinoside | 4.0 | 0/5 | 6.33 ± 0.38 |
| | 5.0 | 3/10 | |
| | 6.0 | 4/10 | |
| | 7.0 | 6/10 | |
| | 8.0 | 4/5 | |
| | 10.0 | 5/5 | |

TABLE 2—*Concluded*

| COMPOUND | DOSE | NUMBER IN SYSTOLE/ NUMBER USED | $SD_{50}$ ± S E. |
|---|---|---|---|
| | *μgm. per gm.* | | *μgm. per gm.* |
| Strophanthidin-β-*l*-arabinoside | 0.20 | 1/10 | 0.308 ± 0.03 |
| | 0.25 | 5/10 | |
| | 0.30 | 5/10 | |
| | 0.35 | 6/10 | |
| | 0.40 | 6/10 | |
| | 0.50 | 8/10 | |
| | 0.70 | 5/5 | |
| Strophanthidin-β-tetraacetyl-*d*-galactoside | 3 0 | 0/2 | 11.29 ± 1.85 |
| | 5.0 | 1/8 | |
| | 10.0 | 2/5 | |
| | 12.5 | 3/5 | |
| | 14.0 | 3/5 | |
| | 20.0 | 4/5 | |
| Ouabain | 0.7 | 0/10 | 0.828 ± 0.02 |
| | 0.8 | 4/10 | |
| | 0.9 | 8/10 | |

The most interesting feature is that two deacetyl-glycosides, namely, strophanthidin-β-*d*-glucoside and -*l*-arabinoside, are not only more potent than strophanthidin, but also more potent than the natural glycoside cymarin. They are the first examples which show the superiority of synthetic cardiac glycosides over the natural ones. The third deacetyl-glycoside, that is, strophanthidin-β-*d*-xyloside, is also decidedly more active than strophanthidin. Its mean lethal dose in cats appears smaller than that of cymarin, but it is not significantly different from the latter. In frogs, however, it is very probably more active than cymarin, since the latter has about the same activity as ouabain (18). There is no question that strophanthidin-β-*d*-xyloside, too, is highly potent, and has at least the same activity as cymarin. In another report (24), it was shown that in cats, at least, deacetyldigilanids A and B were less potent than digilanids A and B, respectively. It is, therefore, not possible to postulate with certainty the favorable influence of deacetylation of the carbohydrate constituent.

## SUMMARY

1. Strophanthidin acetate and 7 synthetic glycosides of strophanthidin all have a digitalis-like action.

2. When assayed in cats and frogs, strophanthidin acetate, strophanthidin-β-*d*-glucoside, -*d*-xyloside, and -*l*-arabinoside, prove more potent than strophanthidin. Strophanthidin-β-tetraacetyl-*d*-glucoside, -triacetyl-*d*-xyloside, -triacetyl-*l*-arabinoside, and -tetraacetyl-*d*-galactoside, on the other hand, are weaker than strophanthidin.

3. Strophanthidin-β-*d*-glucoside and -*l*-arabinoside are also more potent than cymarin, the natural glycoside from which strophanthidin is originally obtained. Strophanthidin-β-*d*-xyloside is at least as active as cymarin.

*Acknowledgment.* The authors are indebted to Dr. Frederick C. Uhle in the preparation of the glycosides, and Messrs. Frank A. Steldt, Robert C. Anderson, E. Brown Robbins, Chester C. Hargreaves, Harold Worth, and William T. Winchester for their valuable assistance in numerous experiments.

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# CORTICAL EFFECTS OF DEMEROL[1]

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Received for publication June 12, 1942

Early experience with the administration of Demerol suggested that in some respects the action of this drug resembles that of morphine, but that the effect on the central nervous system is somewhat different and perhaps more profound. It has been shown (1, 2) that morphine addiction may alter the human electroencephalogram in one of the following ways:

1. An individual showing a low amplitude, low percentage alpha rhythm shows an enhanced alpha rhythm during addition, the change being from one portion of the normal range to another.
2. An individual originally having a good alpha rhythm shows little change during addition, except when the dosage is increasing rapidly beyond the level to which tolerance has been developed. Under these conditions slow waves are seen which disappear if the dose is held constant, and tolerance is allowed to catch up with the dosage.

The study presented here was undertaken in an attempt to determine the cortical effects of repeated doses of Demerol using methods similar to those previously used in studying morphine.

METHODS. Five prisoner patients, serving sentences long enough to insure clinical recovery after withdrawal, volunteered for the study. All of these men had been previously addicted to opiates, but had received no drugs for at least six months, hence records taken before the administration of Demerol are representative of the non-addicted state.

Brain potentials were studied with a four channel electroencephalograph, recording on photographic paper. An observer was always with the patient to record movements and to insure the absence of sleep.

Tremors were recorded from the right index finger, using the photoelectric recording method (3) which permits free finger movement with no appreciable loading.

Demerol was administered in initial doses of 100 mgm. After this first dose each man chose the size and frequency of his dosage to meet his particular desires. Limits of 300 mgm. and 1½ hours were imposed on the dosage.

An electroencephalogram was taken each week throughout the study and at 15 day intervals following withdrawal, until the records returned to the pre-study types. Tremor records were not started until the study was underway, when it became apparent that abnormal tremors were an important part of the drug effect.

RESULTS. All of the patients took the drug at fairly regular intervals in gradually increasing doses. They occasionally reduced their dose voluntarily for short periods, when unpleasant effects developed, but in general they enjoyed the effects sufficiently to ask regularly for larger amounts.

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[1] *1*-methyl-*4*-phenyl-piperidine-*4*-carbonic acid ethyl ester, known in Europe as Dolantin This compound was supplied through the courtesy of Doctor O. W. Barlow of the Winthrop Chemical Company.

[2] Biophysicist, United States Public Health Service.

Each case showed grossly the same clinical picture, varying only in details. The common findings were constipation, exaggerated tremors, graduating into muscle twitches and finally into gross jerking of the extremities, hallucinations, increased sensitivity to sudden noise, and weakness in the extremities. The E.E.G.s also followed the same course in each case. Slow waves were seen early in the study, and became progressively slower and of greater amplitude.

The tremor records showed some rhythms at frequencies not far from normal with large swings occurring at irregular intervals.

The following case histories are typical of the behavior patterns observed. The time in days is calculated from the date on which the administration of Demerol was started. The dosage figures give the total dose in milligrams, and the number of injections for that particular day.

*Case 1.* The pre-study E.E.G., fig. 1 A, shows a normal, low voltage alpha rhythm in all leads, with no evidence of abnormal slow waves.

| *Days* | *Dosage* | |
|---|---|---|
| 3 | 825/9 | Complaining of inability to concentrate. |
| 6 | 1100/11 | Constipated. |
| 12 | 1500/12 | Jerking in sleep. |
| 14 | 1500/11 | E.E.G. showed chiefly slow waves with bursts of high potential waves at 3–4 per second, fig. 1 B. |
| 17 | 1500/10 | Perspiring profusely. |
| 21 | 1650/11 | Nervous, startle reactions to even slight unexpected noise. |
| 28 | | Drug withheld for 22 hours. Abstinence signs of mild intensity. |
| 35 | 1800/10 | Exaggerated, involuntary tremors. E.E.G. showed bursts of slow waves interspersed with some normal activity. Finger tremor showed some frequencies comparable to those recorded from the cortex with large twitches at frequent intervals, fig. 1 D. |
| 47 | 2000/10 | On awakening patient found he had bitten his tongue while asleep, but had no recollection of the incident. |
| 48 | 2200/11 | Bursts of laughter while asleep. |
| 60 | 2860/13 | Twitches and jerks more pronounced. The E.E.G. at this time showed slow waves at 2–3 per second, fig. 1 C. |
| 62 | | Drug withheld for 24 hours. Abstinence signs of moderate intensity. |
| 69 | 3180/15 | Lost consciousness and fell on cement floor. Sustained bruises about head, and body burns from a cup of hot coffee he had been holding. Was unconscious for perhaps five minutes, "I just got paralyzed." |
| 70 | 2980/15 | Unable to move extremities on awakening. Violent jerking of extremities. Was helped to sitting position and finally regained the use of limbs. |
| 71 | | Withdrawal. Marked signs of abstinence notable for the absence of the aching commonly experienced following morphine withdrawal. The slow waves in the E.E.G. persisted for 36 hours after withdrawal. Forty-eight hours after withdrawal there was a decided improvement in the cortical rhythms, fig. 1 E, but an exaggerated tremor persisted. The tremor wave form in fig. 1 E is typical of this period. This tremor persisted for several days but finally both tremor and cortical potentials returned to normal. |

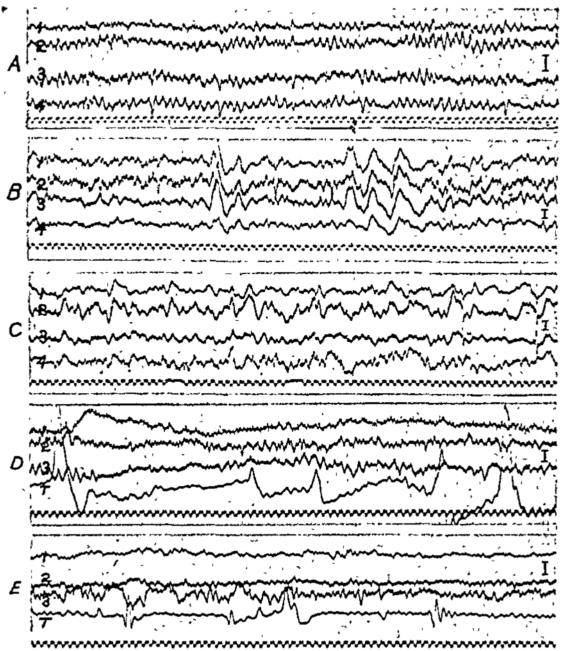

Fig 1. E.E.G s and Tremors Recorded from Case 1

A. Pre-study records.

B. 14 days after start of Demerol. Large slow waves appear from all head regions.

C. 60 days after start of Demerol The large slow waves persist.

D. 35 days after start of Demerol. The slow cortical activity is less pronounced but gross tremor movements occur frequently.

E. Forty-eight hours after withdrawal. There is an improvement in the cortical records but an abnormal tremor is still present.

50 microvolt calibrations. 1, l. frontal; 2, l. central; 3, l. occip.; 4, rt. occip.; T, right index finger tremor.

——————— = 1 sec.

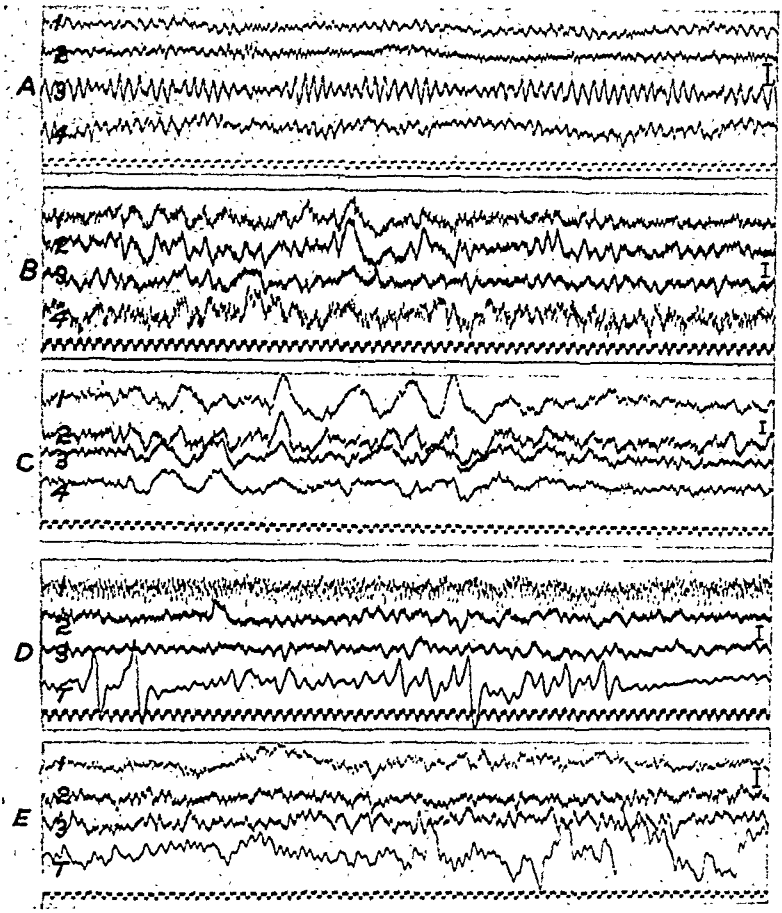

Fig. 2. E.E.G.s and Tremors, Case 2

A. Pre-study records—normal frequencies.
B. 55 days after start of Demerol. Large slow waves in all leads.
C. 67 days after start of Demerol. The rhythms are further slowed.
D. 55 days after start of Demerol. The large tremors have no cortical representation.
E. Twenty-four hours after withdrawal. Slow waves and an abnormal tremor are still present.

50 microvolt calibrations. 1, l. frontal; 2, l. central; 3, l. occip.; 4, rt. occip.; T, right index finger tremor.

———— = 1 sec.

*Case 2.* The preinjection record, fig. 2 A, shows a lack of synchronism between the hemispheres, but only normal frequencies are found.

| *Days* | *Dosage* | |
|---|---|---|
| 4 | 1175/12 | Constipated. "Good medicine." |
| 5 | 1175/12 | Very nervous. |
| 11 | 1675/12 | Twitching while asleep. |
| 13 | 1700/12 | Jerking very pronounced. E.E.G. showed many slow waves. |

| *Days* | *Dosage* | |
|---|---|---|
| 15 | 1700/12 | Talking and moaning while asleep. While walking about ward staggered against the wall and raised his hands to protect his head. Says something hit him on the head. Feelings of claustrophobia. |
| 19 | 1775/11 | Complains of loss of memory. Hallucinations about people touching him. Walks aimlessly about the ward. |
| 28 | | Drug withheld for 22 hours. Abstinence signs of mild intensity. |
| 35 | 1770/10 | Disoriented. Gets up frequently at night and walks aimlessly about the ward. The E.E.G. continued to show predominantly slow wave activity. |
| 41 | 2160/12 | Has difficulty in talking. |
| 52 | 2400/12 | Jumped out of bed with fists up to protect himself from an imagined assailant. Disorientation as to time. |
| 55 | 2660/14 | Disoriented. Wrapped 24 hour urine specimen bottle in a blanket and held electric fan before it to "cool it off." When questioned by the attendants he returned to reality and was much amused at this incident. The E.E.G. at this time showed many large slow waves, fig. 2 B. The tremor showed some rhythms with frequencies approximately equal to the cortical frequency, but with many large amplitude jerks, fig. 2 D. |
| 60 | 2470/13 | Worst twitches and jerks so far. "Head feels numb." |
| 63 | | Drug withheld for 24 hours. Abstinence signs of moderate intensity. Reports an absence of aching but says if he was on the street he would seek Demerol for relief. |
| 67 | 2850/15 | Fell backwards to floor while at urinal. Seized door knob to pull himself up and fell again. Cuts and abrasions about the head, but no signs of cortical damage. The E.E.G. taken a few hours before this incident showed large amplitude slow waves from all leads, fig. 2 C. |
| 70 | | Withdrawal. Marked signs of abstinence. |

Twenty-four hours after withdrawal the E.E.G. showed less slow activity than the preceeding record but had not returned to normal, fig. 2 E. The tremor records continued to show large amplitude movements with some rhythmic tremors.

The results specifically described are typical of the findings in all of the cases. Slow waves appeared in the E.E.G. after only a few days of administration of Demerol. These slow waves became progressively slower, increased in amplitude as the study progressed, and persisted for about forty-eight hours after withdrawal, after which there was a slow return to the original type of record.

It has been shown (3) that in normals the frequency of the finger tremor is almost identical with the cortical frequencies, and that lesions below this level may result in an almost complete dissociation of these frequencies (4). The tremors recorded after repeated administration of Demerol show in general a component having a frequency not far from the frequency of the cortical rhythms. The correspondence is not as close as with normals, however, and there is no cortical component corresponding to the large twitches regularly recorded from the finger.

Discussion. It seems evident that Demerol is a drug which can have a profound effect on the central nervous system. This can be seen both from the case histories and the E.E.G.s. It is true that all of these men were taking large doses compared to the amount which would be given for the clinical relief of pain, since it is probable that in practise the dosage would rarely exceed 100 mgm every 4 hours (5). Nevertheless they were using the drug as it would be used if it were legally available or could be obtained through illicit channels for the support of addiction. In fact if it were freely available it would probably be used to an even greater excess, for all patients frequently asked that the time interval of administration be reduced.

When large regular doses of morphine are administered there are few effects comparable to those described. For short periods, when dosage exceeds tolerance, slow waves are seen in the E.E.G. but these are mild compared to the large amplitude waves seen with Demerol, and are more transient. Furthermore morphine produces no clinical signs similar to those reported here. The periods of loss of consciousness, and the tongue biting, suggest epileptiform attacks, and the E.E.G.s recorded at these times are not inconsistent with this.

Although the tremor rhythms occasionally have frequencies approximately equal to the cortical frequencies the large finger twitches have no representation in the cortical records. The complete dissociation of this component of the tremor suggests that lower centers are strongly involved. These lower centers may in fact be the primary seat of the drug action, for after withdrawal the cortical rhythms return to normal more promptly than does the tremor. The parallelism between the clinical effects of Demerol and cases of Parkinsonism and chronic manganese poisoning suggests that the striate body is involved.

## CONCLUSION

Demerol is a drug which has a profound effect on the central nervous system when used in quantities sufficient to satisfy the desires of addicts. These effects are of such a nature that serious harm might come to those who could obtain the drug in quantities sufficient to meet such requirements. If the drug were freely available abuse might occur, for all patients considered most of the effects pleasant and desirable, and stated that the discomforts following withdrawal were sufficient to discourage voluntary discontinuance.

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# A NOTE ON THE ACTION OF CERTAIN PHENANTHRENE DERIVATIVES ON THE CIRCULATION IN MAMMALS[1]

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An earlier paper (1) reported that a series of phenanthrene derivatives, made in the course of the research program of the Drug Addiction Committee of the National Research Council, had a striking ability to modify the action of the isolated frog heart and the frog heart in situ. The action resembled block, but was not modified by atropine. The present note is offered to record that 9 of the more soluble members of the above-mentioned series have been examined with respect to their action on the mammalian circulation. A list of these 9 substances follows. Reference should be made to the earlier paper for their structural formulae and physical properties.

326. *1*-Dimethylamino-*1*,*2*,*3*,*4*-tetrahydrophenanthrene hydrochloride.
257. *3*-Dimethylamino-*4*-hydroxy-*1*,*2*,*3*,*4*-tetrahydrophenanthrene hydrochloride.
258. *3*-Piperidino-*4*-hydroxy-*1*,*2*,*3*,*4*-tetrahydrophenanthrene hydrochloride.
259. *3*-py-Tetrahydroisoquinolino-*4*-hydroxy-*1*,*2*,*3*,*4*-tetrahydrophenanthrene hydrochloride.
260. *1*-Hydroxy-*2*-dimethylamino-*1*,*2*,*3*,*4*-tetrahydrophenanthrene hydrochloride.
262. *1*-Hydroxy-*2*-py-tetrahydroisoquinolino-*1*,*2*,*3*,*4*-tetrahydrophenanthrene hydrochloride.
150. *3*-[*2*-(Diethylamino)-*1*-hydroxy-ethyl]-phenanthrene hydrochloride.
352. *3*-[*3*-(Diethylamino)-*1*-hydroxy-n-propyl]-phenanthrene hydrochloride.
342. *2*-[*2*-(Diethylamino)-*1*-hydroxy-ethyl]-*9*,*10*-dihvdrophenanthrene hydrochloride.

These are all new substances, and except for #150, which was examined by Eddy (2), have not hitherto been studied with respect to their action on the circulation.

No pharmacodynamic actions specific to the group have been observed. Perfused through the isolated rabbit heart by a technic developed in this laboratory (3) they cause a decrease in resistance to perfusion, decrease in amplitude of contraction, and decrease in rate after larger amounts. In the intact animal (dog) under dial-urethane, intravenous injection of doses up to 5 mg per kilogram is followed by fall in blood pressure, decrease in nose volume, decrease in kidney volume, and a varying response, usually an increase, in the volume of the hind leg. Injection into the leg through the stump of the artery of the opposite side results in a more marked swelling of the leg. The duration of action is brief, similar to that following nitroglycerine injections. The picture resembles that following morphine except that no tachyphylaxis occurs. Further, injection of morphine to production of tachyphylaxis does not modify the action of the phenanthrenes. When tested by the response of the renal vessels to fixed doses of epinephrine (4, 5), there is no adrenolytic effect. Myocardiograph experiments (cats) reveal no important depression of heart muscle. Nothing resembling the block observed in frog hearts has been seen in any of the experiments on mammals.

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[1] Aided by a grant from the David Trautman Schwartz Research Fund.

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# FEDERATION PROCEEDINGS

The Federation of American Societies for Experimental Biology, composed of The American Physiological Society, The American Society of Biological Chemists, The American Society for Pharmacology and Experimental Therapeutics, The American Society for Experimental Pathology, The American Institute of Nutrition and The American Association of Immunologists, has begun (1942) the publication of the Federation Proceedings.

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# THE INFLUENCE OF BILE ON GASTRIC MOTILITY

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The influence of bile on the motility of the alimentary tract has been a subject for study and observation since ancient times (6). The majority of investigators in the past 40 years have centered their attention on the effect of bile on small and large intestinal motility and have placed little or no emphasis on the response of the stomach (1–7). In 1922 Berti and Bernucci (8) reported that the feeding of bile to dogs increased the speed of gastric evacuation. The converse was true when small doses of bile were injected intravenously, but when large amounts were so given an acceleration of gastric emptying resulted. Still and Carlson (9) found that the experimental production of jaundice in dogs diminished the rate and magnitude of gastric hunger contractions and shortened the period of activity. Freude (10) reported that the introduction of a 20% solution of dehydrocholic acid into the stomach of a patient with pylorospasm produced relaxation of the spasm, increased the speed of emptying and increased the tonus of the stomach. Thorner (11) noted that the speed of passage of a barium meal from the stomach was decreased in jaundiced dogs and humans and that the introduction of bile salts into the stomach increased the speed of gastric evacuation in dogs. Van Liere and Northrup (12) reported that the administration of a therapeutic dose (0.42 gram) of dehydrocholic acid given 10 minutes before a barium test meal produced an average decrease of 21.2% in the gastric emptying time of four healthy young adults. Ackerman, Curl and Crandall (13) are inclined to believe, on the basis of their experiments with bile fistula dogs, that bile has a stimulating effect on gastric emptying time after the injection of a fat meal. However, they found that there was an increase in rate of gastric evacuation in bile fistula dogs given a mixed meal.

While studying the effects of feeding whole bile to patients with a variety of lesions, one of us noted (14) that the symptom of anorexia was often relieved. Preliminary investigations of the influence on the dog's gastric motility of whole hog bile and sodium α glycohyodesoxycholate have been reported previously (15). We then determined to carry these investigations further, by noting the responses of the fasting stomach to whole bile of two different origins and to various constituents of bile, and also by studying the effect of whole bile on digestive contractions. This communication contains the results obtained.

METHOD. Five dogs prepared by operation with gastric fistulae after the method of Carlson (16) have been used. After the animals had been trained and conditioned their gastric tonus and motility were measured by the balloon-manometer method. Fifty cc. of air was injected into the balloon and the catheter was connected to a bromoform manometer. The 50 cc. of air usually created a pressure of 3–6 cm. of water. The gastric tonus and motility were then recorded by Patterson's kymographic ink recording method (17).

The whole bile used in the following experiments had been dried by vacuum distillation at low temperatures[1] and then dissolved in water for introduction into the stomach; in some cases natural liquid bile of dogs and pigs was used directly. The bile salts utilized were isolated and purified from the whole biles. The various inorganic salts employed were C. P. reagents and were given in amounts corresponding with those found in the dried bile. A more or less empirical dose usually was utilized, i.e. 0.66 gram of bile in 10 cc. of water (or 2 grams in 30 cc. of water, sometimes 3.3 grams in 50 cc. of water), and the corresponding amount of bile salts or chemicals found in those quantities of bile.

The average lengths of the gastric hunger resting and contraction phases were carefully determined for all of the dogs used in this study. In testing responses during the resting and contraction stages we assured ourselves that the particular phase was fully established and also that the phase was not normally approaching completion. The dogs were fasted 18–25 hours with an average of about 21 hours.

RESULTS. When whole hog, dog, ox or human bile was introduced into the quiescent stomachs of dogs fasted 18–25 hours, typical hunger contractions were immediately produced (fig. 1A). This response was noted in 45 of 55 experiments and it was elicited to the same extent by all of the various whole biles. However, when any of the whole biles were introduced into a dog's stomach during the contraction phase definite but relatively short inhibition of contractions was produced in 30 of 61 experiments. As hog bile contains sodium α-glycohyodesoxycholate in excess of other bile salts and as there is predominance of sodium glyco- and taurocholate in ox and human bile we decided to determine the effect of these salts on the fasting stomach. When any of these salts were injected into the fasting dog's stomach during the resting phase immediate contractions were produced in 34 of 45 experiments, which results essentially paralleled those obtained when using whole bile (fig. 1C). On the contrary, if the bile salts were introduced during the gastric contraction phase inhibition was produced in 17 of 41 instances. This effect, however, was less marked than that obtained with whole bile.

As choline is present in the dried whole bile, the response to this substance, in the concentration found, was tested. No contractions were called forth in 14 instances, and inhibition of contractions was produced approximately as when using bile salts (10 of 26 experiments).

Introduction of plain water to the amount of 10–15 cc. failed to call forth contractions from the gastric resting phase in all of 5 instances (fig. 1B) and caused mild inhibition of gastric hunger contractions in 10 of 32 times attempted. The effect on contractions in the contraction phase is much the same as that obtained with the bile, bile salts and choline, although the bile inhibition occurred somewhat more consistently. All of the whole biles, when introduced into the stomach, produced esentially identical responses. The same statement may be made concerning the various bile salts.

It was then decided to establish the influence on the stomach of certain of those bile components which are present in gallbladder bile in a higher concentration than in blood serum. Previous studies (18) have demonstrated the presence of magnesium, calcium and potassium in dried whole bile in greater concentrations than in serum. As the concentration of magnesium in hog and ox gallbladder bile is 2–3 times higher than in serum its influence on gastric motility was recorded. An amount of magnesium chloride (0.025 gram in 50 cc. of water) corresponding to that found in 50 cc. of liquid whole bile when introduced into the dog's stomach during the resting phase called forth several isolated contractions in three instances, twice produced a drop of tone and in two experiments resulted in no response. When injected into the stomach during the contraction phase transient inhibition and drop in tone resulted in 6 of 7 experiments, this being distinctly more pronounced than the effect of plain water alone. The response to magnesium phosphate and magnesium sulphate in both the resting and contraction phase was essentially the same as that to magnesium chloride.

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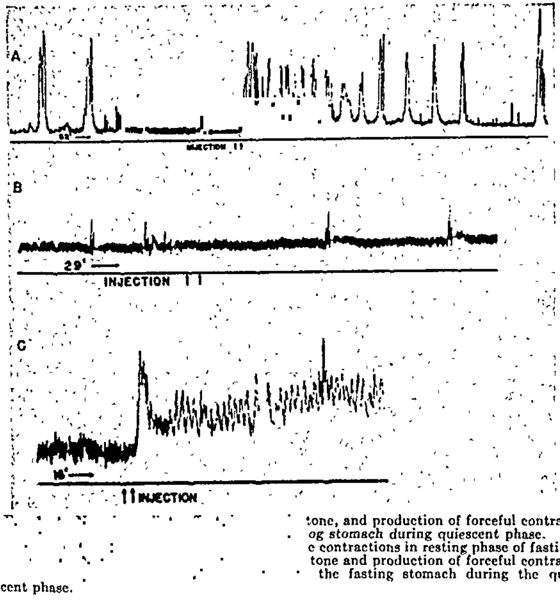
[1] Supplied by Parke, Davis and Company.



tone, and production of forceful contrac-
og stomach during quiescent phase. B.
c contractions in resting phase of fasting
tone and production of forceful contrac-
the fasting stomach during the quiescent phase.

The concentration of calcium in whole hog or ox gallbladder bile is 1½ to 2 times greater than that found in serum. As the amount of calcium in 50 cc. of liquid whole hog or ox bile is approximately 0.0099 gram, the corresponding quantity of calcium chloride (0.027 gram) dissolved in water was introduced into the resting stomach. Occasionally, twice this amount of calcium chloride was used. In 2 instances short periods of contraction resulted and in 6 instances no response was obtained except a slight rise in tone. The effect on the contraction phase was similar to that of magnesium. In 6 of 8 experiments a transient inhibition and drop in tone was observed.

The amount of potassium present in gallbladder bile being 2½ to 4 times greater than in serum, this substance in the form of potassium chloride (0.075 gram in 50 cc. water) was introduced into the stomach. In 5 of 6 experiments potassium chloride initiated hunger contractions in the resting phase which uniformly were identical to those produced by whole bile or bile salts (fig. 2A). After the subcutaneous injection of atropine KCl failed to produce contractions in all of 4 experiments and bile failed to produce contractions in all of 12 experiments (fig. 2B). When potassium was introduced during the contraction phase the tone was diminished and inhibition of contractions occurred in similar fashion as when using plain water in 5 of 8 experiments. The action of sodium chloride on the stomach paralleled that of plain water. In 5 experiments NaCl introduced during the resting phase evoked no contractions; in 3 of 6 experiments contractions were inhibited.

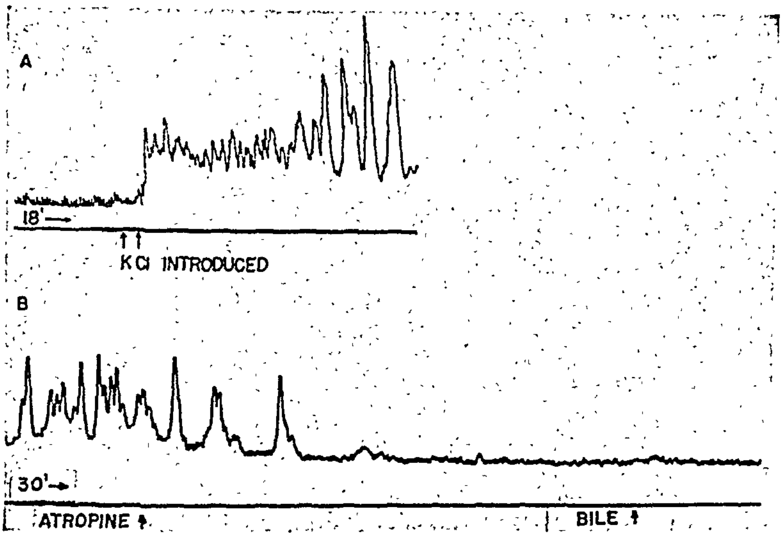


Fig. 2. A. Note immediate production of forceful contractions and increase in tone after introducing potassium chloride into the fasting dog stomach during the quiescent phase. B. Note that the subcutaneous injection of atropine during the gastric contraction phase produced a gastric resting phase during which the introduction of bile into the stomach failed to call forth contractions.

Experiments were then carried out to study the influence of bile on the dog's stomach after the feeding or introduction of bacon, olive oil, gelatin, sugar, canned dog food, potatoes and beef heart. Shortly after the feeding of 100 grams of bacon fat or olive oil hunger contractions ceased. In only 2 of 24 experiments did the feeding of whole bile after the intake of fat call forth weak, transient contractions in the digestion phase produced by fat. Introduction of 300 cc. of concentrated gelatin solution or 25 grams of sugar in 100 cc. of water caused a cessation of gastric hunger contractions in 6 experiments and the subsequent introduction of bile did not effect the response (fig. 3).

Following the injection of mixed dog food into the stomach small irregular digestive contractions occurred and in 6 instances no response was elicited by bile. After the feeding of potatoes slight digestive contractions occurred and in 7 of 10 experiments bile slightly diminished the tone and mildly inhibited contractions. In 3 cases no effect was noted after the introduction of bile.

On the contrary the feeding of 2.27 grams of beef heart, carefully freed of fat and excess connective tissue, called forth digestive contractions similar to Carlson's type I and type II gastric hunger contractions in 12 instances, but sometimes the contractions were reduced in magnitude. This response was similar to that obtained by Mulinos (19) after the feeding of meat though the contractions described by him corresponded more closely to Carlson's type III contractions. The contractions occurred after a 6–8 minute interval of reduced size of contractions and rise of tone following the ingestion of the beef heart. Ox bile and hog bile placed in the stomach after meat feeding diminished the tone and contractions in the majority of instances (in 5 of 8 experiments) but in certain experiments (3 of 8 experiments) a definite rise of tone occurred (fig. 4).

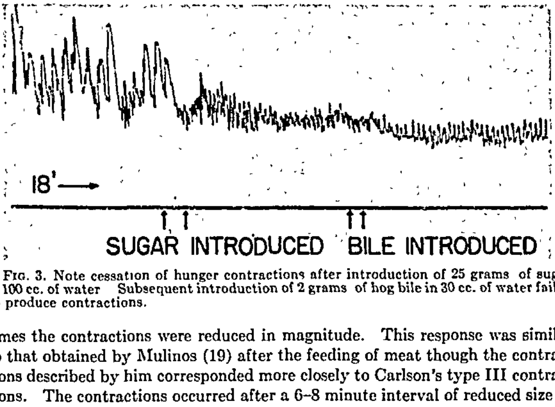


Fig. 3. Note cessation of hunger contractions after introduction of 25 grams of sugar in 100 cc. of water Subsequent introduction of 2 grams of hog bile in 30 cc. of water failed to produce contractions.

It appears that regardless of which kind of bile or bile salt is placed in the fasting dog's stomach during the resting phase, similar responses are obtained. Whole bile, bile salts and potassium chloride produce an immediate contraction phase of long duration, while only occasionally calcium and magnesium salts call forth isolated contractions under these circumstances.

We are unable to state definitely whether the bile effect is exerted through nerve stimulation, but since in 12 experiments the subcutaneous injection of 1 mgm. of atropine during the contraction phase produced a gastric resting phase during which the introduction of bile or potassium failed to elicit contractions, the vegetative nervous system may be the mediator, perhaps by way of cholinergic activity (fig. 2). The action of bile on gastric motility may be either upon the vagal endings directly or indirectly by way of a short inherent reflex mechanism.

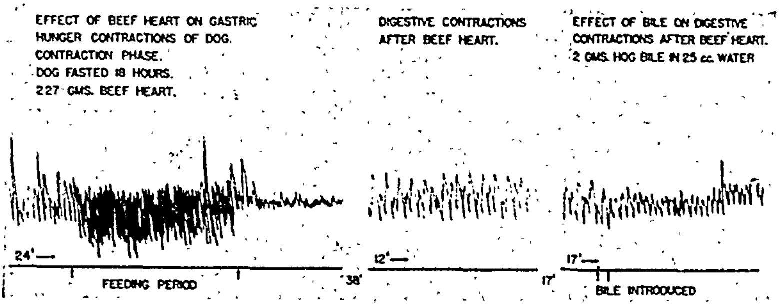


FIG. 4. Note production of type II hunger contractions and rise of tone, 6–8 minutes after the feeding of beef heart. Subsequent introduction of bile produced a rise in tone.

TABLE 1

*Tabulation of results*

| SUBSTANCE INTRODUCED INTO EMPTY STOMACH | NUMBER OF EXPERIMENTS | RESTING PHASE | | CONTRACTION PHASE | | |
|---|---|---|---|---|---|---|
| | | Per cent experiments contractions induced | Per cent experiments no contractions induced | Number of experiments | Per cent experiments contractions inhibited | Per cent experiments inhibition contractions |
| Bile......... | 55 | 81.8 | 18.2 | 61 | 49.2 | 50.8 |
| Water.... . | 5 | 0 | 100.0 | 32 | 31.2 | 68.8 |
| Bile salts.... | 45 | 75.5 | 24.5 | 41 | 41.4 | 58.6 |
| Choline..... | 14 | 0 | 100.0 | 26 | 38.5 | 61.5 |
| NaCl..... .. | 5 | 0 | 100.0 | 6 | 50.0 | 50.0 |
| KCl.... . | 8 | 83.3 | 16.7 | 8 | 37.5 | 62.5 |
| $MgCl_2$ .... | 7 | 42.9* | 57.1† | 7 | 85.7 | 14.3 |
| $CaCl_2$.. . .. | 8 | 25.0‡ | 75.0 | 8 | 75.0 | 25.0 |

* Isolated contractions only.
† In twenty-eight 5% drop of tone.
‡ Few contractions only.

However, one must be cautious in drawing conclusions from the results of the atropine experiments, since the pharmacologic gastric resting phase provoked by atropine seems to be somewhat different from the physiologic gastric resting phase normally occurring between hunger contraction phases in the stomachs of fasting dogs. The physiologic resting phase is less stable and is of shorter duration than the atropine-induced resting phase. Nevertheless, it seems justifiable to consider that the vagal components play an important part in the mechanism of the bile effects, since atropine prevents them. As bile frequently is present in the healthy stomach, it is possible that its influence on motility may produce sensations of hunger.

The results are summarized in table 1.

## SUMMARY

1. When certain dried whole biles or bile salts are placed in a fasting dog's stomach during the resting phase, gastric hunger contractions are produced.
2. When the foregoing substances are placed in a fasting dog's stomach during the contraction phase, transient inhibition occurred in slightly more than half of the experiments. This result roughly parallels the findings using water.
3. Choline, in a concentration found in dried bile, does not call forth contractions from the quiescent phase and produces inhibition of contractions approximately as did bile.
4. Several magnesium salts produced occasional contractions during the resting phase in approximately 43% and caused inhibition of contractions in 85%.
5. Potassium chloride, on the other hand, produced definite contractions during the quiescent phase and diminished the tone during the contraction phase.
6. The feeding of certain foods such as fat, sugar and gelatin markedly inhibited hunger contractions and only occasionally did bile call forth contractions in the resting phase thus induced.
7. The ingestion of beef heart produced contractions similar to hunger contractions but these contractions at times were of reduced magnitude.
8. Neither the mechanism of the bile effect nor the exact substance producing this effect are known, but suspicion that the vagal components are involved seems warranted since atropine obliterates the effects of bile and certain of the bile components.

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# ACUTE AND CHRONIC EFFECTS OF PENTACHLOROPHENOL AND SODIUM PENTACHLOROPHENATE UPON EXPERIMENTAL ANIMALS[1]

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The effectiveness of pentachlorophenol and its sodium salt as preservative agents has led to their widespread employment in solutions for the treatment of wood and other products, and their use in various types of food containers and wrappers is contemplated. An appraisal of the hazard involved in the handling of these toxic compounds in technical dipping operations requires a knowledge of the local effects of single or repeated contacts of the substances with the skin, as well as of the rate and degree of their absorption following such exposures. The concentrations permissible in food containers can be determined only after exact information on their cumulative action has become available.

Attention was first called to the toxicity of pentachlorophenol by Bechhold and Ehrlich, in 1906 (1). More extensive studies were carried out by Kehoe, Deichmann and Kitzmiller in 1939 (2) and by Boyd, McGavack, Terranova and Piccione in 1940 (3) and 1941 (4).

This paper summarizes (A) the acute toxic effects (most of which were reported in detail in 1939 (2)), (B) local and systemic effects resulting from repeated cutaneous applications, (C) cumulative effect of small oral doses, (D) distribution of pentachlorophenol in the tissues, (E) detoxification of the compound by conjugation, and (F) effective removal of pentachlorophenol accidentally spilled upon the skin. The histopathological observations carried out in conjunction with these studies will be reported in a separate paper.

Experimental procedure employed. Unless otherwise indicated, the hair of albino rabbits was clipped very short over an area of about 20 square inches on the belly, in preparation for skin applications. After an application an animal was kept for 2 hours under mild but efficient restraint, and then placed in a cage. The compound was not washed off.

Since the studies on the distribution of pentachlorophenol in blood and tissues involved the withdrawal of blood samples from the heart at frequent intervals, it was necessary to employ several rabbits for each dosage. Blood was withdrawn from different animals at the successive periods in such order that the animal taken for the first sample was not used again until blood had been taken from each of the others.

The rats employed were born and raised in the laboratory from a colony received from the Wistar Institute. The rabbits were bought from a local breeder. The cats used for the feeding experiments were stray animals, and were kept under observation for one month before being used.

Various solvents were employed for pentachlorophenol but sodium pentachlorophenate was always used in aqueous solution. All dosages have been expressed in terms of pentachlorophenol.

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[1] Presented at the meeting of the American Society for Pharmacology and Experimental Therapeutics in Boston, April, 1942.

(A) SUMMARY OF ACUTE TOXIC EFFECTS. Pentachlorophenol or its sodium salt, when absorbed in sufficient quantity, produced in all species of animals studied (dogs, rabbits, rats, guinea pigs), an acute toxic state characterized by increased blood pressure, hyperpyrexia (104–114°F.), hyperglycemia and glycosuria, hyperperistalsis, an increased and later a diminished urinary output, and rapidly developing motor weakness. In addition to these signs and symptoms, dying animals showed complete collapse and asphyxial convulsive movements. Rigor mortis was immediate and profound. The post-mortem evidences of injury were not specific and consisted largely of extensive damage to the vascular system, with heart failure and involvement of the parenchymatous organs. Pentachlorophenol applied cutaneously caused a more or less pronounced edema of the skin, which in about a week became dry and wrinkled. Slight cracks developed and hair was lost completely from the treated areas, but the hair follicles and the deeper structures of the skin apparently suffered no permanent injury. Tables 1 and 2 summarize the minimal concentrations of these compounds lethal for rabbits, and give the $LD_{50}$ dosage for rats.

(B) LOCAL AND SYSTEMIC EFFECTS RESULTING FROM REPEATED CUTANEOUS APPLICATION OF PENTACHLOROPHENOL AND SODIUM PENTACHLOROPHENATE. To simulate the hazards attending the use of dipping mixtures, repeated cutaneous applications of pentachlorophenol or sodium pentachlorophenate in various solvents were made upon the skin of rabbits, and the effects were observed. Some of the preparations were administered daily, others once or twice per week.

(*1*) A one per cent solution of pentachlorophenol in mineral oil was applied to the skin of two rabbits in doses of 10 cc., corresponding to about 40 mgm. per kilogram. At the end of four hours the excess material was wiped off with cotton and, without further washing, the animals were returned to their cages. Both animals survived 21 successive daily treatments without illness, loss of weight or injury to the skin.

(*2*) Doses ranging from 10 to 50 mgm. of pentachlorophenol per kilogram, as a 4% solution in Stanolex Fuel Oil, were applied to the skin of the back of rabbits once or twice a week, for periods ranging from 6 to 61 weeks. The compound was not washed off. The local effects produced were the same as those induced by a single large dose. A rise in rectal temperature of 2–3°F. (measured 8 hours after treatment) occurred occasionally after a dose of 10 mgm. per kilogram, and regularly after larger doses. Erythrocyte counts, differential counts, and hemoglobin determinations were made monthly. The fluctuations observed did not exceed those which occurred in untreated animals. There were no significant changes in body weight. Notwithstanding the absence of specific signs of poisoning, 8 of the 20 rabbits died during the experiment. Table 3 summarizes the dosages and the periods of treatment. Several rabbits were killed for pathological examination; the gross and histopathological tissue changes were in direct relation to the size of the dose and the period of treatment.

(*3*) Similar experiments were made with the sodium salt (2% solution). On 32 successive days a rabbit was given the dose of 63 mgm. per kilogram. At no time did this animal show illness or injury to the skin. Another rabbit

## TABLE 1

*The toxicity of pentachlorophenol and sodium pentachlorophenate for rabbits (single administration)*

| COMPOUND | CONCENTRATION AND SOLVENT | LETHAL DOSES IN TERMS OF PENTACHLOROPHENOL | TIME TILL DEATH | COMMENTS |
|---|---|---|---|---|
| Cutaneous administration | | | | |
| | | *mgm./kgm.* | *hrs.* | |
| Pentachlorophenol | 5% in olive oil | | | 180 mgm./kgm. produced no apparent ill effects |
| Pentachlorophenol | 5% in 95% ethyl alcohol | | | 150 mgm./kgm. produced no apparent ill effects |
| Pentachlorophenol | 10% in 95% ethyl alcohol | | | 1111 mgm./kgm. produced acute illness and local damage |
| Pentachlorophenol | 11% in olive oil | | | 450 mgm./kgm. produced acute illness |
| Pentachlorophenol | 10% in corn oil | | | 326 mgm./kgm. produced no apparent ill effects |
| Pentachlorophenol | 5% in Stanolex fuel oil no. 1 | 60–70 | 1½–4 | |
| Pentachlorophenol | 5% in Shell dione oil | 110–120 | 5–6½ | |
| Pentachlorophenol | 5% in Std. oil (Ind.) pale paraffin oil | | | 150 mgm./kgm. produced acute illness and local damage |
| Pentachlorophenol | 5% in Stanolex furnace oil | 90–100 | 1½–3 | |
| Pentachlorophenol | 5% in Shell no. 3 fuel oil | 130–170 | 6 | |
| Pentachlorophenol | 1.8% in pine oil | 40–50 | 9 –22 | |
| Na pentachlorophenate | 10% aqueous | 250 | 3 –8 | |
| Oral administration | | | | |
| Pentachlorophenol | 5% in Stanolex fuel oil | 70–90 | 2 –5 | |
| Pentachlorophenol | 11% in olive oil | 100–130 | 10 –16 | |
| Na pentachlorophenate | 5% aqueous | 250–300 | 3 –6 | |
| Subcutanous administration | | | | |
| Pentachlorophenol | 5% in olive oil | 70–85 | 3 –6 | |
| Na pentachlorophenate | 10% aqueous | 100 | 7 | |
| Intravenous administration | | | | |
| Na pentachlorophenate | 2% in water | 22–23 | 1½–4 | |

received two doses (on 2 successive days) of 113 mgm. per kilogram. This animal died four hours after the second treatment. A third rabbit died from acute intoxication after the thirteenth application of 111 mgm. per kilogram, applied over a period of 43 days.

TABLE 2

*The toxicity of pentachlorophenol and sodium pentachlorophenate for rats (single administration)*

| COMPOUND | CONCENTRATION AND SOLVENT | NUMBER OF RATS USED | $LD_{50}$ | TIME TILL DEATH |
|---|---|---|---|---|
| Oral administration | | | | |
| | | | *mgm/kgm.* | *hrs.* |
| Pentachlorophenol | 0.5% in Stanolex fuel oil | 80 | 27.3 | 3–19 |
| Pentachlorophenol | 1% in olive oil | 60 | 77.0 | 3–11 |
| Na pentachlorophenate | 2% in water | 60 | 210.6 | 2–13 |
| Subcutaneous administration | | | | |
| Na Pentachlorophenate | 2% in water | 80 | 66.3 | 2–8 |

TABLE 3

*Results of repeated application of a 4% solution of pentachlorophenol in Stanolex fuel oil to the skin of rabbits*

| NUMBER OF RABBITS EMPLOYED | DOSE OF PENTACHLOROPHENOL | NUMBER OF APPLICATIONS PER WEEK | DURATION OF TREATMENT | TOTAL DOSE OF PENTACHLOROPHENOL APPLIED | FATE |
|---|---|---|---|---|---|
| | *mgm/kgm* | | *weeks* | *mgm./kgm.* | |
| 3 | 10 | 1 | 36 | 360 | Killed |
| 1 | 10 | 2 | 47 | 940 | Died |
| 1 | 10 | 2 | 47 | 940 | Killed |
| 4 | 10 | 2 | 60 | 1200 | Died |
| 3 | 35 | 2 | 8 | 560 | Killed |
| 1 | 35 | 2 | 8 | 560 | Killed |
| 1 | 50 | 1 | 5 | 250 | Died |
| 1 | 50 | 1 | 27 | 1350 | Killed |
| 1 | 50 | 1 | 13 | 650 | Died |
| 1 | 50 | 1 | 42 | 2100 | Killed |
| 1 | 50 | 1 | 12 | 600 | Died |
| 1 | 50 | 1 | 47 | 2350 | Killed |
| 1 | 50 | 1 | 6 | 300 | Killed |

(4) Ten cc. doses of a 1% solution of the sodium salt (about 40 mgm./kg.) were applied daily (except Sundays) for 100 consecutive days to the skin of 6 rabbits. In the case of 3 rabbits the solution was allowed to remain on the skin for 30 minutes, and in 3 others for one hour, after which the compound was washed off with soap and water. The treated areas occasionally showed mild irritation but no wrinkling or cracking of the skin and no loss of hair. The gain in body weight was normal, and there were no fatalities.

(*5*) Before attempting to apply any inferences drawn from these data to the problem of the hazards involved in repeated contact with dipping solutions, it appeared desirable to secure more definite information on the extent of absorption of these compounds than that afforded by skin changes, the occurrence of slight temperature elevations, or by the intervention of death without signs of acute intoxication. This could best be secured by determining the pentachlorophenol concentration in the blood after varied degrees of exposure, and comparing this concentration with that required to cause the appearance of symptoms or fatalities. The analytical method employed for the estimation of pentachlorophenol (6) is based on the formation and spectrophotometric determination of a reddish-yellow pigment produced by the action of fuming nitric acid on pentachlorophenol. The method was first applied to the blood of rabbits following a single cutaneous application, for 30 or 60 minutes, of 100 mgm. of the sodium salt (1% solution). The determination was made upon 20 cc. samples obtained by pooling 5 cc. of heart blood from each of 4 treated rabbits, the blood having been withdrawn 1, 4 and 6½ hours following the treatment. There was no indication of absorption of the compound. However, the repeated application of a 1% solution of the sodium salt to the skin of rabbits does induce absorption to a degree that is demonstrable by this method. Two of the 4 rabbits treated with 100 mgm. daily for 100 days were killed and the tissues pooled and examined (table 4). The blood of the first pair contained 0.45 mgm.% of pentachlorophenol and the urine 6.62 mgm.%. In the blood of the latter pair, allowed but half as much time for absorption, the corresponding values were 0.23 and 4.93 mgm.%. Additional small quantities were recovered from the kidney, liver, lung and muscle. None of these rabbits had shown signs of illness or local irritation.

TABLE 4

*Concentration of pentachlorophenol in tissues of the rabbit after cutaneous application of 10 cc. of a 1 per cent aqueous solution of the sodium salt for 100 consecutive days, except Sundays*

| TISSUE | *Rabbits 4264 and 4265* COMPOUND WASHED OFF 1 HR. AFTER APPLICATION | *Rabbits 4261 and 4262* COMPOUND WASHED OFF 30 MIN. AFTER APPLICATION |
|---|---|---|
| | Pentachlorophenol (*mgm./100 grams sample*) | |
| Urine | 6.62 | 4.93 |
| Blood | 0.45 | 0.23 |
| Kidney | 0.26 | 0.14 |
| Lung | 0.09 | 0.14 |
| Liver | 0.05 | 0 |
| Brain | 0 | 0 |
| Muscle | 0 | 0.06 |

(For purposes of comparison, a series of determinations were made of the blood concentrations occurring at the time of the appearance of symptoms and at death following administration of lethal oral doses. The results (table 5) indicate that at the time the temperature had risen from 2 to 2.6°F., the blood concentrations had attained values of from 4.5 to 8.0 mgm.%. These values remained at about this level when death occurred, although the temperature had then risen from 4.4 to 9.2°F. above the normal.) The blood concentrations encountered at the time of the appearance of symptoms were from 10 to 17.7 times as high as those resulting from 100 daily applications of the 1 per cent solution.

(C) The cumulative effect of small oral doses. Since the use of these compounds in food containers is contemplated, it is essential to learn whether repeated small oral doses can produce a chronic or acute toxic state as a result of cumulative action. This problem has been attacked by a study of the concentration of pentachlorophenol in the blood following single or repeated small oral doses, and by prolonged feeding experiments.

TABLE 5

*Concentration of pentachlorophenol in the blood of rabbits at the time of appearance of symptoms and at death after a minimal lethal oral dose of pentachlorophenol*

| NO. OF RABBIT | RECTAL TEMPERATURE BEFORE TREATMENT | AT APPEARANCE OF SYMPTOMS | | AT DEATH | |
|---|---|---|---|---|---|
| | | Rise in temp. above control | Pentachlorophenol in blood | Rise in temp above control | Pentachlorophenol in blood |
| | °F. | °F. | *mgm./100 cc.* | °F. | *mgm./100 cc.* |
| 6396 | 104.6 | 2 | 8.00 | | |
| 6392 | 104.4 | 2.2 | 6.25 | 8.2 | 6.75 |
| 6391 | 104.8 | 2.2 | 6.50 | 8.3 | 6.75 |
| 6394 | 104.6 | 2.4 | 4.50 | 5.4 | 5.75 |
| 6395 | 104.8 | 2.4 | 4.75 | 4.4 | 8 50 |
| 6393 | 103.8 | 2.6 | 6.00 | 9.2 | 4.00 |

(*1*) *Blood and tissue concentrations following single oral doses.* The sodium salt was administered orally to rabbits in dosages equivalent to 5, 18, 37, 92 and 185 mgm. of pentachlorophenol per kilogram. The results of the observations on these animals are shown in fig. 1. Absorption started almost immediately after a dose equivalent to 18 mgm. per kilogram or more. The blood level reached a peak only after about seven hours when a dose of 37 mgm. per kilogram was administered. About four days elapsed before all of the compound disappeared from the blood after the administration of 37 mgm. per kilogram.

(*2*) *Blood and tissue concentrations following repeated small oral doses.* An analogous type of experiment was carried out on 23 rabbits each of which was given 90 doses (on successive days excepting Sundays) of the sodium salt in the form of a 0.1% solution, each dose being equivalent to 3 mgm. of pentachlorophenol per kilogram. None of the animals showed signs of acute poisoning, but some died as the result of accidents.

The analytical data are summarized in figure 3 where it may be seen that feeding at this level caused an accumulation of the compound in the blood. The

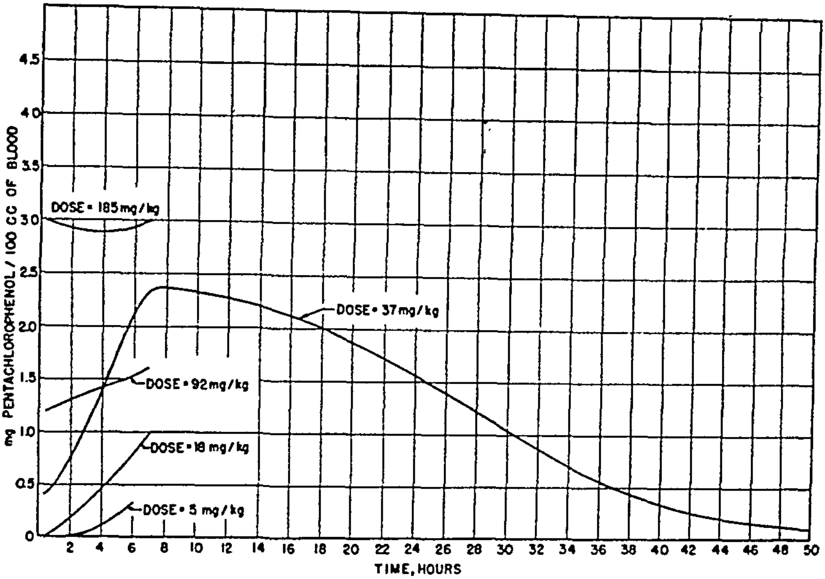

FIG. 1. CONCENTRATION OF PENTACHLOROPHENOL IN THE BLOOD OF RABBITS FOLLOWING A SINGLE ORAL DOSE OF SODIUM PENTACHLOROPHENATE

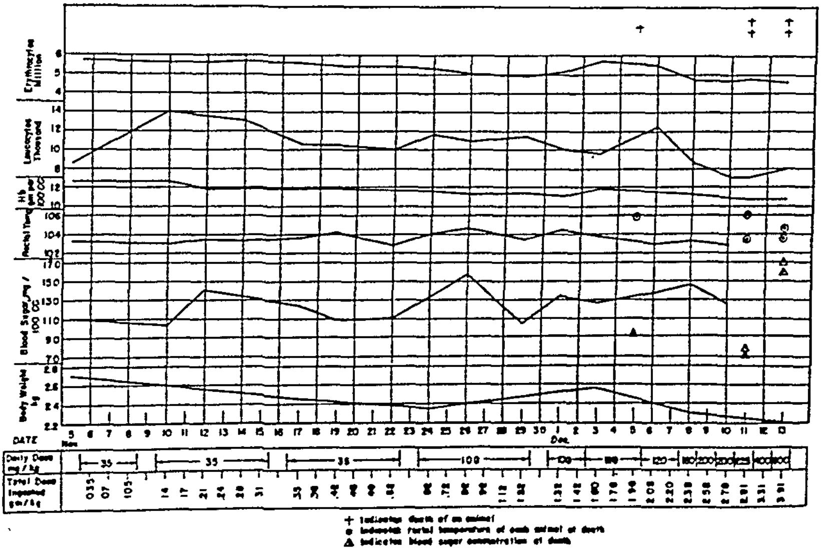

FIG. 2. EFFECTS OF REPEATED ORAL DOSES OF SODIUM PENTACHLOROPHENATE ON ERYTHROCYTE AND LEUKOCYTE COUNTS, CONCENTRATION OF HEMOGLOBIN AND BLOOD SUGAR, AND ON THE BODY WEIGHT AND RECTAL TEMPERATURE IN RABBITS

average peak concentration of about 0.6 mgm.% was reached in about 4 days. Then, *for the remaining 86 days, the blood concentration remained about the* same. The range of all observations was between 0.3 and 1.0 mgm. pentachlorophenol per 100 cc. of blood. The blood level found in these animals is comparable with that (0.45 mgm.) found in the blood of animals given 100 daily *skin applications of 100* mgm. each.

(3) *Feeding experiments.* Experiments were performed to detect any alterations in the well-being of animals of various species which might result from the daily ingestion of these compounds.

*Rats.* Pentachlorophenol, dissolved in a small quantity of 95% ethanol, was added to Purina Fox Chow pellets and fed to two groups of 10 rats each. *Group 1* was fed over a period of 26 weeks, each rat ingesting daily approximately 5 mgm. of the compound in 8.5 grams of food. These animals neither gained nor lost weight during this period. Their failure to grow apparently resulted from a reduction in their intake of food. *Group 2* was treated for 28 weeks, each of these rats consuming daily about 3.9 mgm. of pentachlorophenol in 13 grams of food. Although these animals doubled their weight, *they failed* to gain at the normal rate. This failure may also be attributed to an inadequate consumption of food. All animals were anesthetized with ether and killed by bleeding from the large cervical vessels. Post-mortem examination of their tissues revealed no gross and only insignificant histological abnormalities.

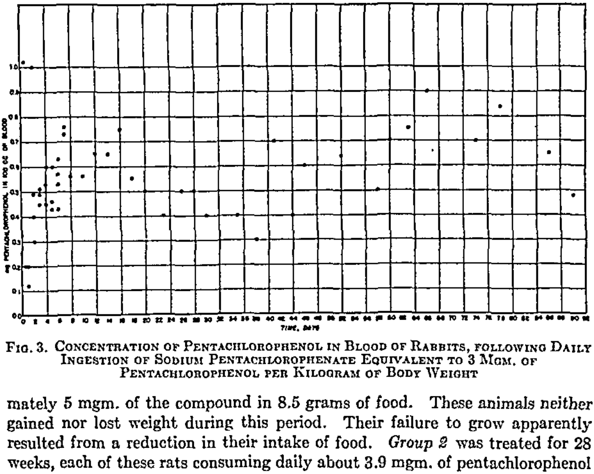

FIG. 3. CONCENTRATION OF PENTACHLOROPHENOL IN BLOOD OF RABBITS, FOLLOWING DAILY INGESTION OF SODIUM PENTACHLOROPHENATE EQUIVALENT TO 3 MGM. OF PENTACHLOROPHENOL PER KILOGRAM OF BODY WEIGHT

*Cats.* Difficulty in securing adequate consumption of food (boiled salmon and ground meat to which pentachlorophenol had been added) was again encountered when cats were used. The animals refused to eat when the content of pentachlorophenol was sufficiently high to give a daily intake *of 5–10 mgm.*

per kilogram. Therefore smaller amounts were used. Four cats received pentachlorophenol or its sodium salt in doses equivalent to 1.25 and 2.5 mgm. per kilogram. This diet was fed for about 10 weeks. The animals showed some loss of appetite and loss of weight, but none of the cats displayed signs of pentachlorophenol poisoning. They were killed with ether 20 hours after the last meal; at this time the blood contained from about 0.3 to 1.8 mgm. of pentachlorophenol per 100 cc.

*Rabbits.* To form some idea of the degree of tolerance which might develop, each of five rabbits was given a daily oral dose of 35 mgm. pentachlorophenol per kilogram (about $\frac{1}{8}$ of the lethal dose) as a 0.5% solution of the sodium salt, for 15 days. During the following 19 days a 5% solution was used and the dose was raised gradually to 600 mgm. per kilogram (about twice the lethal dose). Rectal temperatures, blood counts and determinations of hemoglobin and of blood sugar (Folin micro method) were made every second or third day, six hours after the treatment. The animals were weighed weekly.

One animal died after having ingested a total of 1.9 grams of pentachlorophenol per kilogram; two died after the ingestion of 2.9 grams per kilogram, and two others after 3.9 grams per kilogram. It may be seen from fig. 2 that this severe treatment caused loss of body weight, a very slight reduction in the number of erythrocytes and a parallel drop in the concentration of hemoglobin. Leukocytes and rectal temperatures fluctuated but did not exceed the limits of variations observed in control rabbits.

The most definite evidence of the establishment of tolerance to the compound is the finding of blood concentrations of 14, 21, 22, 36 and 39 mgm.% of pentachlorophenol in animals that died. After single lethal doses of pentachlorophenol, the concentration of pentachlorophenol in the blood of rabbits did not exceed 8.5 mgm.% (table 5).

(D) DISTRIBUTION OF PENTACHLOROPHENOL IN THE TISSUES. (*1*) The tissues of 6 rabbits that died accidentally in 2 to 7 days after the start of the experiment in which 3 mgm. of pentachlorophenol per kilogram was fed (cf. Section C 2), were analyzed. The results indicate that blood concentrations of 0.4 to 0.8 mgm.% of pentachlorophenol are associated with concentrations ranging from 0.15 to 0.4 mgm.% in the liver and from 0 to 0.3 mgm.% in the kidney.

(*2*) Complete results on the distribution of pentachlorophenol in the tissues were obtained in the case of 2 rabbits. Each was given an oral dose of the sodium salt (0.25% sol.) equivalent to 37 mgm. of pentachlorophenol per kilogram and then was put into a metabolism cage for 24 hours, after which it was sacrificed for analysis of the tissues. Of the administered quantities of pentachlorophenol 92 and 91% respectively were recovered. The bulk of the material was found in the urine (tables 6 and 7) and about 4 and 7%, respectively, were present in the gastroenteric tract. The remainder was well distributed throughout the tissues. Obviously in the rabbit the kidney is the principal route of elimination of pentachlorophenol from the body, a fact which suggests that urinary concentrations may offer the best means of determining the severity of human exposure.

TABLE 6

*Distribution of pentachlorophenol in the tissues of rabbit D-6730, 24 hours after oral administration of sodium pentachlorophenate in one dose (94 mgm. expressed as pentachlorophenol)*

| TISSUE | TOTAL WEIGHT OF TISSUE | PCP IN 10 GRAMS SAMPLE ANALYZED | PCP IN TOTAL TISSUE | PCP RECOVERED |
|---|---|---|---|---|
| | *grams* | *mgm.* | *mgm.* | *per cent of amt ingested* |
| Urine and feces | 199.1 | 3.40 | 66.21 | 70.6 |
| Stomach and intestine (wall and contents) | 575.7 | 0.11 | 6.33 | 6.76 |
| Muscle | 931.0 | 0.065 | 6.05 | 6.42 |
| Bones | 382.6 | 0.075 | 2.86 | 3.06 |
| Skin | 260.9 | 0.08 | 2.08 | 2.22 |
| Blood | 222.6 | 0.08 | 1.78 | 1.90 |
| Liver and gall bladder | 100.0 | 0.14 | 0.70 | 0.743 |
| Kidneys | 17.7 | 0.185 | 0.25 | 0.267 |
| Heart, lungs and testes | 25.9 | 0.06 | 0.16 | 0.171 |
| Central nervous system | 12.8 | 0.075* | 0.09 | 0.096 |
| Total recovery | | | 86.51 | 92.23 |

* Entire sample of 12.8 grams analyzed.

TABLE 7

*Distribution of pentachlorophenol in the tissues of rabbit D-6750, 24 hours after oral administration of sodium pentachlorophenate in one dose (97 mgm. expressed as pentachlorophenol)*

| TISSUE | TOTAL WEIGHT OF TISSUE | PCP IN 10 GRAM SAMPLE ANALYZED | PCP IN TOTAL TISSUE | PCP RECOVERED |
|---|---|---|---|---|
| | *grams* | *mgm.* | *mgm.* | *per cent of amt. ingested* |
| Urine | 182.5 | 3.70 | 67.53 | 70.3 |
| Feces | 10.0 | 0.30 | 0.30 | 0.3 |
| Stomach and intestine (wall and contents) | 380.0 | 0.10 | 3.80 | 3.9 |
| Muscle | 1003.0 | 0.035 | 3.50 | 3.6 |
| Bones | 344.3 | 0.035 | 1.20 | 1.25 |
| Skin | 382.0 | 0.10 | 3.82 | 3.91 |
| Blood | 239.2 | 0.23 | 5.50 | 5.7 |
| Liver and gall bladder | 82.9 | 0.235 | 1.95 | 2.0 |
| Kidneys | 18.2 | 0.2 | 0.36 | 0.38 |
| Heart, lungs and testes | 31.0 | 0.10 | 0.31 | 0.32 |
| Central nervous system | 12.5 | 0.085* | 0.085 | 0.088 |
| Total recovery | | | 88.35 | 91.1 |

* Entire sample of 12.5 grams analyzed.

*(3)* To throw further light on the excretion of pentachlorophenol, 2 animals were given oral doses of 25 mgm. pentachlorophenol per kilogram (0.5% solu-

tion of the sodium salt), and 2 others, doses of 50 mgm. per kilogram. The excreta were then collected for periods ranging from 7 to 12 days, and analyzed. The data are given in table 8. From ½ to ⅔ of the dose was excreted in the urine and by far the greatest proportion (37 to 50% of the total dose) was excreted in the first 24 hours after administration. From 1 to 4% was excreted in the feces. The proportion of the dosage recovered in the urine of these animals was somewhat less than in the preceding experiments, probably because a more concentrated solution was given to the latter animals.

TABLE 8

*The excretion of pentachlorophenol*

In urine and feces of rabbits after oral administration of single dose of sodium pentachlorophenate equivalent to 25 or 50 mgm. pentachlorophenol per kilogram.

| | RABBIT 6551 | | RABBIT 6716 | | RABBIT 6552 | | RABBIT 671 | |
|---|---|---|---|---|---|---|---|---|
| | Total amount administered | | | | | | | |
| | 71 mgm. | | 65 mgm. | | 127 mgm. | | 135 mgm. | |
| | Cumulative percentage | | | | | | | |
| | Urine | Feces | Urine | Feces | Urine | Feces | Urine | Feces |
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Excreted 1st day | 44.5 | 0.3 | 50.0 | —* | 37.4 | 0.3 | 44.9 | 0.2 |
| Excreted 2nd day | 55.0 | 0.9 | 58.4 | — | 47.2 | 1.3 | 45.7 | 0.2 |
| Excreted 3rd day | 62.6 | 1.6 | 60.2 | 0.2 | 50.1 | 1.7 | 46.6 | 0.5 |
| Excreted 4th day | 64.4 | 2.3 | 61.1 | 0.2 | 51.1 | 2.0 | 47.0 | 0.5 |
| Excreted 5th day | 65.0 | 2.8 | 61.3 | 0.2 | — | 2.1 | 47.3 | 0.5 |
| Excreted 6th day | 65.6 | 3.4 | 61.7 | 0.2 | 51.7 | 2.3 | 47.5 | 0.7 |
| Excreted 7th day | 66.1 | 4.0 | 62.0 | 0.6 | 52.2 | 2.4 | 47.5 | 0.7 |
| Excreted 8th day | | | 62.7 | 0.6 | 52.8 | 2.7 | 47.5 | 1.0 |
| Excreted 9th day | | | 63.2 | 0.8 | | | 47.5 | 1.0 |
| Excreted 10th day | | | 63.2 | 0.8 | | | 47.7 | 1.0 |
| Excreted 11th day | | | 63.2 | 0.8 | | | 47.7 | 1.0 |
| Excreted 12th day | | | 63.2 | 0.8 | | | 47.7 | |
| Total percentage recovered in urine and feces | 70.1% | | 64.0% | | 55.5% | | 48.7% | |

* — = No sample.

(*4*) From the data on the recovery of administered pentachlorophenol (tables 6 and 7) it would appear that approximately 9% of an oral dose of 37 mgm. per kilo is broken down in the tissues of the rabbit in 24 hours. To determine the rate of destruction more accurately, a series of rats were given intraperitoneal doses (2.4 mgm.) of the salt. The animals were kept in individual glass beakers, in which all their excreta were collected, and were sacrificed at intervals. The total carcass of each was ground, mixed and analyzed, the accumulated excreta being analyzed separately. About 12.5% of the injected pentachlorophenol was excreted in the first 24 hours, and about 40% was broken down during the first day. The material was not completely lost from the body at the end of the

6th day, about 13% being still present (in this respect the rat resembles the rabbit, fig. 1). The results are summarized in table 9 and in figure 4. Most of the compound found in the urine and feces was excreted during the first 24 hours.

TABLE 9

*Rate of destruction and recovery of pentachlorophenol in the carcass and excreta of the rat following a single intraperitoneal dose of 2.4 mgm. per animal*

| TIME BETWEEN TREATMENT AND DEATH | PCP FOUND IN EXCRETA | | PCP FOUND IN CARCASS | | PCP DESTROYED | |
|---|---|---|---|---|---|---|
| | *mgm.* | *Per cent of amount injected* | *Mgm.* | *Per cent of amount injected* | *Mgm.* | *Per cent of amount injected* |
| Killed immediately | 0 | 0 | 2.40 | 100 | 0 | 0 |
| 3 hours | 0.18 | 7.5 | 1.98 | 82.5 | 0.24 | 10.0 |
| 12 hours | 0.16 | 6.6 | 1.64 | 68.3 | 0.60 | 25.1 |
| 24 hours | 0.30 | 12.5 | 1.13 | 47.0 | 0.97 | 40.5[a] |
| 2 days | 0.40 | 16.6 | 0.55 | 22.9 | 1.45 | 60.5 |
| 3 days | 0.32 | 13.3 | 0.44 | 18.3 | 1.64 | 68.4 |
| 4 days | 0.20 | 8.3 | 0.35 | 14.5 | 1.85 | 77.2 |
| 5 days | 0.23 | 9.5 | 0.30 | 12.5 | 1.87 | 78.0 |
| 6 days | 0.28 | 11.6 | 0.32 | 13.3 | 1.80 | 75.1 |

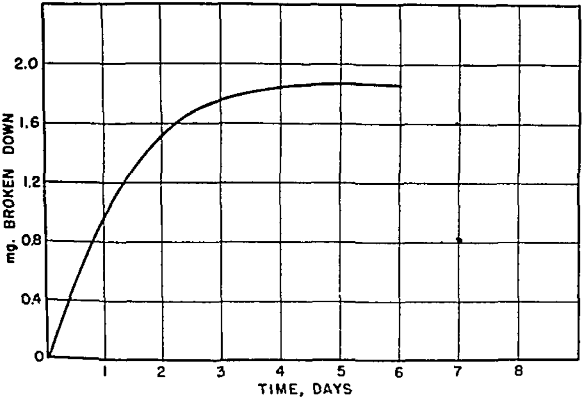

FIG. 4. RATE OF DESTRUCTION OF PENTACHLOROPHENOL IN THE RAT (SINGLE INTRAPERITONEAL DOSE OF 2.4 MGM. PER ANIMAL)

(E) THE DETOXIFICATION OF PENTACHLOROPHENOL BY CONJUGATION. An effort was made to learn whether, as in the case of phenol or cresol poisoning, a clinical criterion of absorption might be found in a diminished excretion of the inorganic urinary sulfates (6) or in an increased output of glucuronic acid (7) as a result of conjugation. It was found, however, that only traces of pentachlorophenol are conjugated.

(F) EFFECTIVE REMOVAL OF PENTACHLOROPHENOL FROM THE SKIN. A 2.5% solution of pentachlorophenol in Stanolex Fuel Oil was dropped from a syringe upon the back of each of a series of rabbits in amounts sufficient to cover about one-sixth of the total body area. The animals were returned to their cages and after various periods of time were washed with soap and water or with alcohol. All four of the animals survived that were washed with alcohol at 30 minutes after the application, but 2 rabbits, allowed to go for 45 minutes before washing, died, as did 2 others that were not washed until 60 minutes elapsed. All 9 rabbits washed with soap and water after 45 minutes or less survived, but all 4 that went unwashed for 60 minutes died. Soap and water is therefore more effective for the removal of the compound, besides being cheaper and more readily available than alcohol.

## SUMMARY

1. Pentachlorophenol and sodium pentachlorophenate, when absorbed in sufficient quantity into the tissues of dogs, rabbits, rats and guinea pigs, produce an acute toxic state characterized by accelerated respiration, moderately elevated blood pressure, hyperpyrexia, hyperglycemia and glycosuria and hyperperistalsis (vomiting was observed in dogs after a subcutaneous dose). The urinary output is at first increased, later diminished, and there is a rapidly developing motor weakness which in fatal cases terminates in asphyxial convulsions and cardiac and muscular collapse. Rigor mortis is immediate and profound.

2. Irritation of the skin and marked local damage followed by complete recovery is the usual result of cutaneous application of single or repeated doses of pentachlorophenol in fuel oils. Single and repeated applications of aqueous solutions of the salt may cause mild local damage.

3. A gradual loss of weight but no significant changes in the hemoglobin content of the blood are noted in rabbits given repeated, slightly sublethal doses of the salt. The numbers of erythrocytes and of various types of leukocyte remain within normal limits. Rectal temperatures and blood sugar values rise markedly after the administration of single sublethal doses but fail to show such elevations when the doses are repeated.

4. Absorption of sodium pentachlorophenate through the skin of the rabbit cannot be detected by blood analyses following a single one-hour application of 100 mgm. of this salt. Repeated applications of this quantity result in demonstrable absorption, the blood level rising to about 0.45 mgm.%. (This is approximately one-fifteenth of the concentration of pentachlorophenol encountered in the blood at the time of the appearance of symptoms following a lethal oral dose.)

5. Absorption of sodium pentachlorophenate from the stomach of the rabbit starts almost immediately after administration of a single dose of 18 mgm. per kilogram, reaching a peak of 2.4 mgm.% in the blood (with a dose of 37 mgm./kg) in about 7 hours. Four days elapse before all traces of the compound dis-

appear from the blood. The daily administration to rabbits (by stomach tube) of quantities of the salt equivalent to 3 mgm. of pentachlorophenol per kilogram leads to a blood level of about 0.6 mgm.%. Feeding of the salt (mixed with food) in a dosage of 3.9 mgm. per rat per day apparently causes no tissue damage but results in inadequate consumption of food, presumably because of the unpleasant taste which the compound imparts to the diet. Cats refuse food altogether when it contains about 5 mgm. pentachlorophenol per 100 gram of salmon or meat. A tolerance develops in rabbits when sublethal oral doses are administered repeatedly.

6. Within the limits of dosages employed, approximately 70% of orally administered sodium pentachlorophenate is excreted in the urine by the rabbit (the bulk of it in the first 24 hours); about 9% is broken down, and about 6% is in the gastrointestinal tract at the end of the first day, the remainder being well distributed throughout the tissues. Very small amounts are excreted with the feces. In the rat, following the intraperitoneal injection of 40 mgm. per kilogram only about 13% of the amount administered is excreted, 40% is broken down, and about 47% remains in the tissues at the end of the first day. Pentachlorophenol is not detoxified by conjugation with sulfuric or glucuronic acid.

7. Soap and plenty of water is a more effective agent than alcohol for the removal of pentachlorophenol or sodium pentachlorophenate from the skin.

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# PHARMACOLOGY OF COLOUR REGULATION IN AMPHIBIA AND THE IMPORTANCE OF ENDOCRINE GLANDS

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The colour of amphibia depends on the degree of dispersion of the pigment in the skin melanophores and is regulated by endocrine and nervous mechanisms in which the hypophysis and adrenals play an important part (1, 2, 6, 7, 10, 14). The influence of chemical agents on colour of amphibia, and the importance of hypophyseal secretion in the action of drugs, have been extensively studied by Fuchs (4), Hogben (6), Houssay and Ungar (7), Giersberg (5), Smith (15), Leszczynski (9), Shen (13), Teague, Noojin and Geiling (18), with some disagreement in results which may be explained by the different species of amphibia used in experiments. According to Chang and Associates (3), Parker and Rosenblueth (12), Spaeth and Barbour (16) the melanophores are autonomic effectors which, in their responses to chemical mediators, are adrenergic in some species, adrenergic and cholinergic in others. In this paper experiments are reported on the action of several drugs on the colour of the toad *Bufo arenarum* Hensel and the frog *Leptodactylus ocellatus* (L) Gir., with special reference to the rôle of the hypophysis and the adrenals.

Methods. Toads weighing 100–150 gm. and frogs weighing 50–80 gm. of both sexes were used. Changes in the pigmentary effector system were recorded according to the color observed directly and to the distribution of the pigment in the melanophores. The following indices of colour and dispersion of melanic-pigment in melanophores were used: *1*, pale green colour, concentrated melanic pigment; *2*, light green, scantily dispersed melanic pigment; *3*, green colour moderately dispersed melanic pigment; *4*, dark green, largely dispersed melanic pigment; *5*, black colour, melanic pigment in reticulum. Melanophores were observed in the deep layers of the skin with a microscope with oblique illumination, obj. 8–20, oc. 8 (fig. 1).

The substances were dissolved in saline solution (0.6%) in such a proportion that the doses employed were contained in 1 or 2 cc. They were injected intraperitoneally through the dorsum. In some cases (acetylcholine) drugs were injected into the carotid artery by means of a very thin needle or a perfusion was done through the arteries of the isolated hind limbs.

Some substances were also tested by immersing isolated fragments of skin in the corresponding solutions. Extracts of bovine posterior lobe of hypophysis were prepared by treating the dry powder with NaOH 0.1 N during 3 minutes at 100° C., filtering and discarding the precipitate. Hypophysectomy was performed by oral approach, following Giusti and Houssay's technique and adrenal destruction was carried out by electrocoagulation.

Results. The results of the numerous experiments performed with each substance are summarized in tables 1, 2 and 3. The two numbers in brackets express the colour and melanophore index at the beginning and at the end of the experiment; E.P.L.H. means extract of hypophyseal posterior lobe; F. 933 means piperidine methil *3*-benzo dioxane.

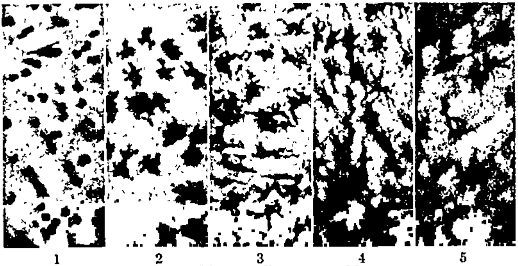

FIG. 1 DISPERSION OF MELANIC PIGMENT IN MELANOPHORES
1, concentrated; 2, scantly dispersed; 3, moderately dispersed; 4, largely dispersed; 5, reticulum.

TABLE 1

*Action of drugs on the colour of the frog Leptodactylus ocellatus (L) Gir. (normal or hypophysectomized)*

| SUBSTANCE | AMOUNT INJECTED | ACTION ON | | REMARKS |
|---|---|---|---|---|
| | | Normal | Hypophysectomized | |
| Adrenaline | 0.5-1 mg. | Pales (5-1) | No action | F.933 reverses, cocaine reinforces |
| Calcium chloride | 75-50 mg. | Pales (5-1) | No action | F.933 inhibits, cocaine reinforces |
| Ephedrine | 10 mg | Pales (5-1) | No action | F.933 reverses in normal and hypophysectomized frog |
| Cocaine | 10 mg | Pales (3-1) | No action | |
| Nicotine | 3 mg. | Darkens (1-5) | Darkens (1-4) | |
| Caffeine | 15 mg. | Darkens (1-5) | Darkens (1-4) | |
| F.933 in whole animal | 2.5-15 mg. | Darkens (1-5) | Darkens (1-3) | Additional effects with E.P.L.H. |
| F.933 perfusion of isolated hind limbs | 1% | Darkens (1-4) | Darkens (1-4) | Potassium is necessary to produce darkening |
| Veratrine | 0.5-1 mg. | Darkens (1-5) | Darkens (1-5) | |
| Potassium chloride | 25-50 mg. | Darkens (1-5) | No action | Hypophysectomized darkens if previously injected with F.933 (0.5 mg.) |
| Atropine | 10 mg. | Darkens (1-4) | Darkens (1-3) | |
| Acetylcholine | 10 mg. | No action | No action | With or without previous injection of eserine |
| Eserine | 30-20-10 mg. | Darkens (1-4) | No action (1-1) | |

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Results. The results of the numerous experiments performed with each substance are summarized in tables 1, 2 and 3. The two numbers in brackets express the colour and melanophore index at the beginning and at the end of the experiment; E.P.L.H. means extract of hypophyseal posterior lobe; F. 933 means piperidine methil *3*-benzo dioxane.

barium; *S*, producing a diphasic action, first paling and then darkening the colour like nicotine or caffeine (in the toad). This action varies somewhat according to the species since in *Leptodactylus ocellatus* (L) Gir., caffeine and nicotine only produce darkening of skin. The rôle of hypophysis in the effects of drugs on this system has been the object of many studies. There have been some discrepancies which may be referable to species differences, since F. 933, veratrine, nicotine and atropine have no action on the colour of the hypophysectomized *Bufo arenarum* Hensel while the same drugs intensely darken the colour of the hypophysectomized *Leptodactylus ocellatus* (L) Gir. (figs. 2 and 3).

The action of drugs on the melanophores of hypophysectomized amphibia varies with the drug as well as the species. Thus, the colour of the pale hypophysectomized toad is not modified by the injection of atropine, veratrine or F. 933, but is darkened by caffeine or barium. On the other hand in the hypophysectomized *Leptodactylus ocellatus* (L) Gir., potassium, eserine and acetylcholine have no action upon colour, while veratrine, F. 933, atropine and caffeine produce a deep darkening of the skin (figs. 2 and 3).

TABLE 3

*Action of drugs on the colour of the toad Bufo arenarum Hensel (normal or adrenalectomized)*

| SUBSTANCE | AMOUNT INJECTED | ACTION ON | | REMARKS |
|---|---|---|---|---|
| | | Normal | Adrenalectomized | |
| | mg. | | | |
| Adrenaline | 2-1 | Pales<br>(5-1) | Pales<br>(5-1) | F.933 inhibits, weak antagonism with E.P.L.H. |
| Cocaine | 4 | Pales<br>(5-1) | Weak paling action<br>(5-4) | F.933 inhibits |
| Ephedrine | 10 | Pales<br>(5-1) | Weak paling action<br>(5-4) | F.933 inhibits or reverses |
| Calcium chloride | 100 | Pales<br>(5-1) | Weak paling action<br>(5-4) | F.933 inhibits |
| Nicotine | 3 | Pales<br>(5-1) | Weak paling action<br>(5-4) | F.933 inhibits the paling action |
| Caffeine | 10 | Pales<br>(5-3) | No action | F.933 inhibits the paling action |

The colour of amphibia may be modified in several ways by chemical agents: *a*, through a direct action on the melanophore, such as can be observed with barium and caffeine in the toad by perfusion experiments, by their action on isolated pieces of skin, or by their injection in hypophysectomized animals; *b*, by reinforcing the action of the melanotropic hormone, as can be observed in the hypophysectomized toad with F. 933 A small dose of this substance alone has no action on colour but it increases considerably the melanic-dispersing action of extract of the posterior lobe of the hypophysis. An action of drugs on the rate of secretion of melanotropic hormone as described by Shen (13) in *Rana temporaria* has not been observed in our experiments. In no case were clear-cut changes of

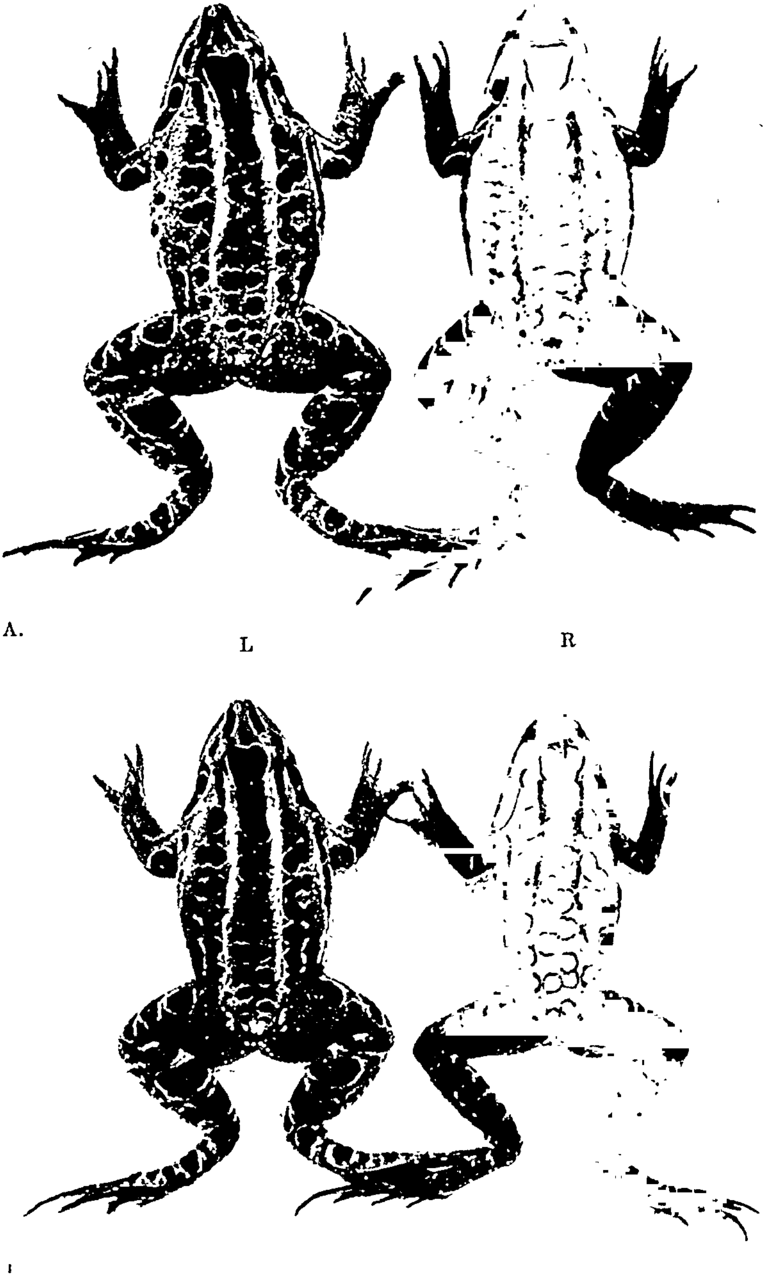

FIG. 2. A: L, normal control frog; R, normal frog injected with F.933. B: L, hypophysectomized control frog; R, hypophysectomized frog injected with F.933.

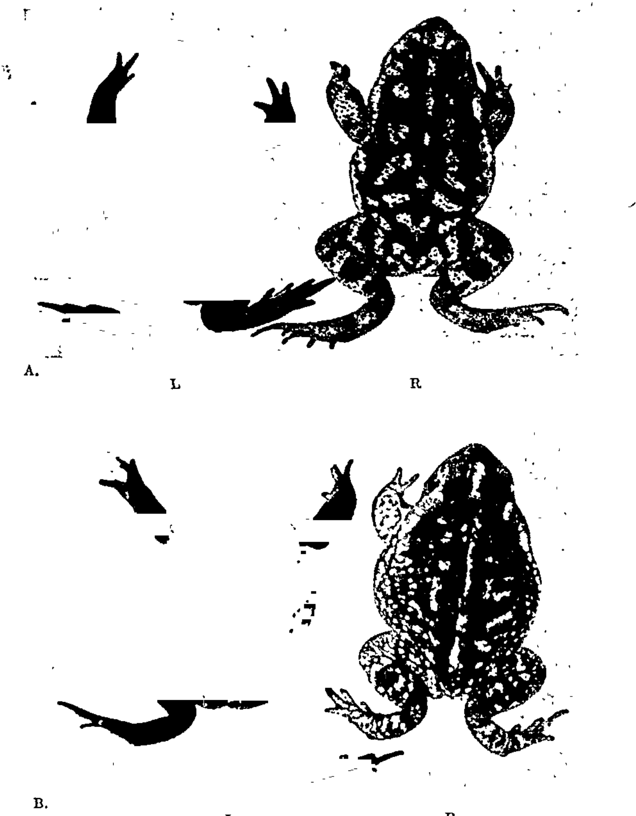

FIG. 3. A: L, normal toad injected with F.933, R, hypophysectomized toad injected with F 933.

B: L, hypophysectomized toad injected with caffeine; R, hypophysectomzed control toad colour of toad and *Leptodactylus ocellatus* (L) Gir., obtained by applying on the diencephalo-hypophyseal region small plugs of cotton soaked with a 1% solution of F. 933, veratrine, nicotine, acetylcholine or potassium. Finally, drugs can modify the colour of amphibia by combinations of some of the mechanisms already described. Thus the mechanism through which chemical agents change the state of the pigmentary effector system seems to be a very complex one.

The adrenals play a considerable rôle in modifying the action of those chemical agents that pale the colour of the toad. When the adrenals are destroyed, cocaine, ephedrine, nicotine, caffeine and calcium lose much of their paling action on colour.

The melanophores of *Bufo arenarum* Hensel and *Leptodactylus ocellatus* (L) Gir., are exclusively adrenergic effectors. Whether by injection in hypophysectomized animals by perfusion through the hind limbs or testing the substances in isolated pieces of skin, acetylcholine and eserine, acting simultaneously or successively, have no dispersing action of the melanic pigment. This agrees with our experiments (17) in which excitation of autonomic nerves with stimuli of different intensity and frequency (1 to 160 per second) demonstrated the existence of exclusively melanic-concentrating fibres, whose action is reinforced by cocaine and inhibited by F. 933.

## SUMMARY AND CONCLUSION

The pharmacology of colour changes in *Bufo arenarum* Hensel and in the frog *Leptodactylus ocellatus* (L) Gir., has been comparatively studied, with the following results:

1. Drugs can act upon colour of amphibia; paling it like adrenaline, ephedrine, cocaine, calcium; darkening it like F. 933, veratrine, potassium and barium; or producing a diphasic action like caffeine and nicotine in the toad. This action varies somewhat according to species since caffeine and nicotine have an exclusively darkening action in the *Leptodactylus ocellatus* (L) Gir.
2. The hypophyseal hormone has a fundamental action in the darkening effect of numerous drugs which varies according to species and substance; F. 933, veratrine, and nicotine darken the colour of the hypophysectomized frog but have no action on the colour of the hypophysectomized toad.
3. The ability of melanophores to expand their pigment in the absence of melanotropic hormone varies according to species and drug. Caffeine and barium darken the colour of the hypophysectomized toad, while F. 933, veratrine and atropine have no action. In the hypophysectomized *Leptodactylus ocellatus* (L) Gir., veratrine, F. 933 and atropine darken the colour of the skin.
4. The adrenal secretion is of utmost importance in the paling action on the colour of toad produced by calcium, nicotine, cocaine and ephedrine.
5. The melanophores of *Bufo arenarum* Hensel and of *Leptodactylus ocellatus* (L) Gir., are exclusively adrenergic effectors.

The author is deeply indebted to Professor B. A. Houssay for his advice and criticism throughout this work.

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# THE EFFECTS OF SOME NITRATE ESTERS OF XANTHINE DERIVATIVES[1]

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The esters of nitrous and nitric acids used in therapy for their property of relaxing smooth muscle have been esters of alcohols of simple nature, in themselves not particularly active pharmacologically. Since xanthine derivatives also have the property of relaxing smooth muscle, nitrous and nitric esters of xanthine derivatives might be expected to combine the characteristic actions of both types of substances.

The compounds shown in table 1 were prepared by J. H. Speer at G. D. Searle & Co., Chicago, Ill. The preëminent action of these esters of nitrous and nitric acids is relaxation of vascular smooth muscle. Their vasodepressor effect was measured in five etherized dogs, in comparison with nitroglycerine. Table 1 shows the reciprocal activity ratios in terms of the amounts by weight producing equal falls in arterial pressure. The fall in arterial pressure produced by the intravenous injection of these substances was accompanied by acceleration of cardiac and respiratory rates, as is seen with nitroglycerine. The peripheral site of the vasodilator action was demonstrated by injection into the femoral artery of four dogs in which a constricting clamp had been applied, proximal to a branch in which pressure was measured. Under such circumstances a fall in this pressure in the face of constant systemic arterial pressure indicates local vasodilation. Figure 1 shows that *8*-chloro-*7′*-hydroxycaffeine nitrite and nitrate are more active than equimolecular amounts of sodium nitrite, caffeine, and nitroglycerine. In two experiments in barbitalized dogs the volumes of a 50 cm. segment of small intestine, one hind leg and one kidney were recorded plethysmographically. Fifteen intravenous injections of caffeine, nitroglycerine and *8*-chloro-*7′*-hydroxycaffeine nitrite and nitrate were made. The results, which were qualitatively identical in all cases, were that the fall in arterial pressure was associated with an increase in leg volume and a decrease in volume of small intestine and kidney, which suggests that with these substances active vasodilation occurred in the leg. Figure 2 typifies these experiments.

The perfused isolated rabbit's heart (9 experiments) was used for determining the cardiac effects of these substances. The cannula of Rössler (1) was used to insure passage of all the perfusing fluid through the coronary vessels; this flow was measured by a differential manometer (2). The apex of the heart was connected with an isotonic lever. Figure 3 indicates that in doses of 0.1 mgm. the nitrous and nitric esters have predominantly a vasodilator action. Both are more potent than caffeine, sodium nitrite, and nitroglycerine. If the dose is

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[1] *This investigation was aided by a grant from G. D. Searle and Company, Chicago, Illinois.*

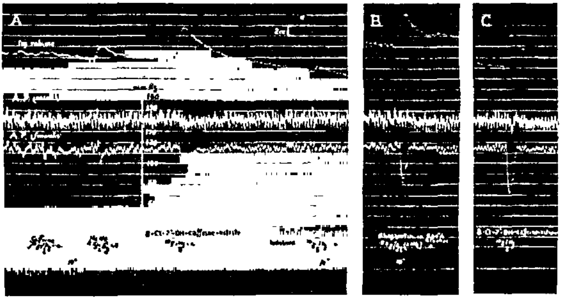


FIG. 1. Dog, barbitalized From the top down, leg volume, carotid arterial pressure, femoral arterial pressure, signal of injection, 10 second intervals. Injections into the femoral artery, in micrograms per kilogram, of: A. Caffeine 70, sodium nitrite 2.5, 8-Cl-7'-OH-caffeine nitrite 10, the same after boiling. B. Nitroglycerine 8.3. C. 8-Cl-7'-OH-caffeine nitrate 10. These are equimolecular amounts except for caffeine which is ten times the molecular equivalent.

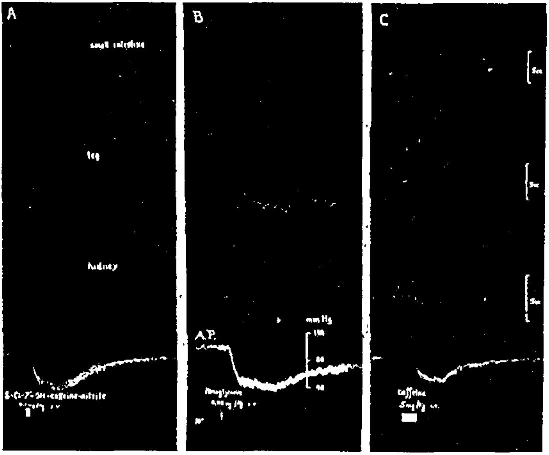


FIG 2 Dog, barbitalized From the top down, volumes of small intestine, leg, and kidney, carotid arterial pressure, signal of injection, 10 second intervals. Injections into [illegible] itroglycerine [illegible] amounts of

increased to 1 mgm., the muscular stimulant action appears (fig. 4). This dose was of necessity dissolved in propylene glycol, which itself had no action. In the mammalian heart-lung preparation, in a dose of 1 mgm., the *8*-chloro-*7'*-hy-

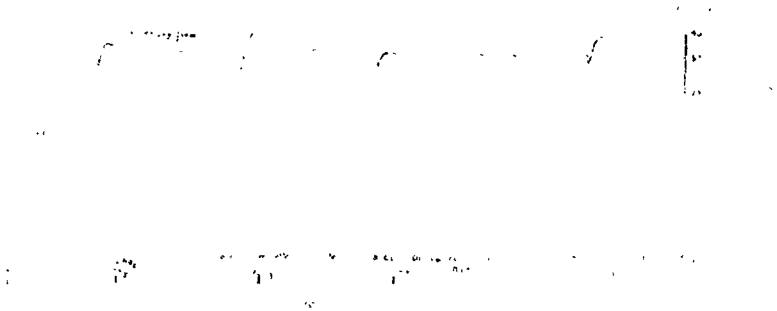
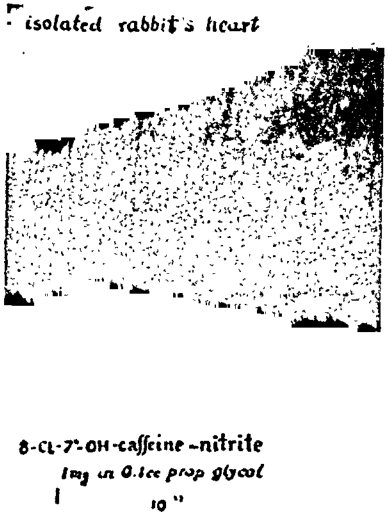


FIG. 3. Isolated rabbit's heart. From the top down coronary flow, ventricular contractions, signal of injection, 10 second intervals. Injections into the aortic cannula, in micrograms, of: caffeine 70, sodium nitrite 25, *8*-Cl-*7'*-OH-caffeine nitrite 100, the same after boiling, *8*-Cl-*7'*-OH-caffeine nitrate 100. These are equimolecular amounts. The downstroke in the record of coronary flow is an artifact produced by the injection.



G. 4. Isolated rabbit's heart. *8*-Cl-*7'*-OH-caffeine nitrite 1 mgm. in 0.1 cc. propylene l injected into aortic cannula. Ten second intervals.

ycaffeine nitrite ester causes a fall in venous pressure and reduction in tolic ventricular size, indicative of a muscular stimulant action.

5 dogs the chlorohydroxycaffeine nitrite and nitrate esters had characternitrate action on the pressure of the cerebrospinal fluid, as seen in figure 5.

They have no influence on the spinal flexion reflex when given intravenously in the largest doses which their vasodepressor action permits. Water diuresis in trained unanesthetized dogs was found to be inhibited by chlorohydroxycaffeine nitrate. Sodium nitrite has this action in man (3). Unlike theophylline, 8-chlorotheophylline, the product of hydrolysis of chlorohydroxycaffeine nitrate *in vitro*, was found to have no effect on urine flow in rabbits under chloral hydrate anesthesia.

The possibility of formation of the above-mentioned chlorotheophylline *in vivo* was examined. Although it can be recovered from urine after its addition, none could be obtained from the urine of dogs receiving chlorohydroxycaffeine nitrate.

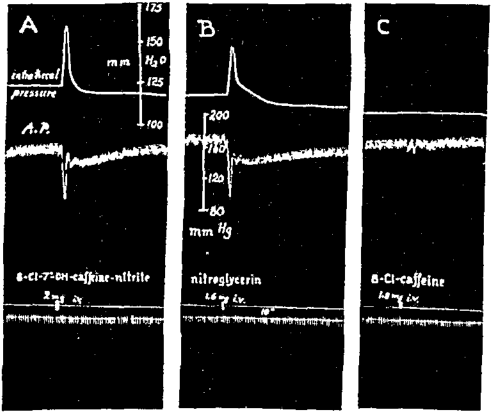
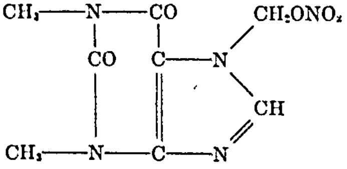
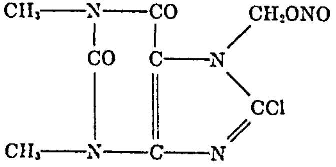
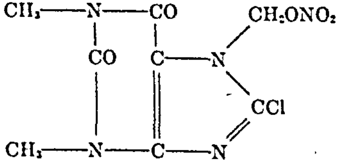


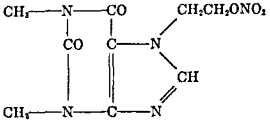
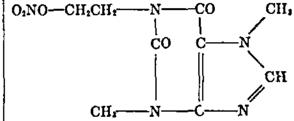
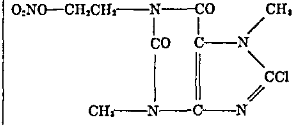
FIG 5. Dog, chloralose anesthesia
carotid arterial pressure, signal of in
femoral vein of: A. 8-Cl-7'-OH-caffeir
C. 8-Cl-caffeine 1 8 mgm. These are equimolecular amounts.

*Action in unanesthetized animals.* In trained dogs, the cardiac and respiratory rates, mean pressure in the femoral artery by needle-puncture (4), skin temperature of the lateral surface of the thigh by Stewart's method (5), and rectal temperature, were studied under the influence of the chlorohydroxycaffeine nitrate and nitrite esters, and erythrityl tetranitrate, all administered by mouth in capsules. The results seen in figure 6 indicate the peripheral vasodilator action of these substances.

The effect of repeated oral administration of small doses of chlorohydroxycaffeine nitrate ester was observed in four trained dogs. The mean femoral

TABLE 1

| NAME | STRUCTURAL FORMULA | RECIPROCAL ACTIVITY RATIO | MORTALITY RATIOS, ORAL ADMINISTRATION TO RATS (MGM. PER KGM.) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 150 | 250 | 300 | 500 | 1000 | 1500 | 2000 |
| Nitroglycerine | $O_2NOCH_2—(O_2NO)CH—CH_2ONO_2$ | 1 | | | | | | | |
| 7′-hydroxycaffeine nitrate |  | 0.25 | 0/3 | 3/3 | | 2/2 | 2/2* | | |
| 8-chloro-7′-hydroxycaffeine nitrite |  | 1 | | | 0/4 | 1/8 | 5/8 | 6/6 | 3/3 |
| 8-chloro-7′-hydroxycaffeine nitrate |  | 0.5 | | | | 0/6 | 5/8 | 6/6 | 4/4 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *1,3*-dimethyl-7-(*2*-hydroxyethyl)xanthine nitrate |  | 10 | | | | 1/4 | 4/4* | | |
| *1*-(*2*-hydroxyethyl)-*3,7*-dimethylxanthine nitrate |  | 20 | 0/3 | 1/3 | | 2/2* | | | |
| *1*-(*2*-hydroxyethyl)-*3,7*-dimethyl-*8*-chloroxanthine nitrate |  | 20 | | | | | 1/4 | 4/4* | |

Mortality ratio = dead animals/animals used.

* Small amount of compound precluded the use of a larger number of animals.

arterial pressure was measured by Parkins' method (4), over a 12 day period before giving the drug, for 35 days of administration, and over 16 days after administration. The substance was given in gelatin capsules at 8:30 a.m., 1:30 and 6:30 p.m. Arterial pressure was measured at 3:30 p.m. Two dogs received at each dose 30 mgm./kgm., one 10 mgm./kgm., and one 3 mgm./kgm. The results are seen in figure 7. One dog on the highest dose vomited occasionally throughout the treatment; no other signs of intoxication were observed. The fall in arterial pressure was not maximal with the largest dose until the fourth or fifth day; at the smallest dose no fall appeared until the sixteenth day. Definite tolerance appeared in one dog receiving the largest dose. The arterial pressure returned slowly to normal after cessation of administration, indicating accumulation of the drug.

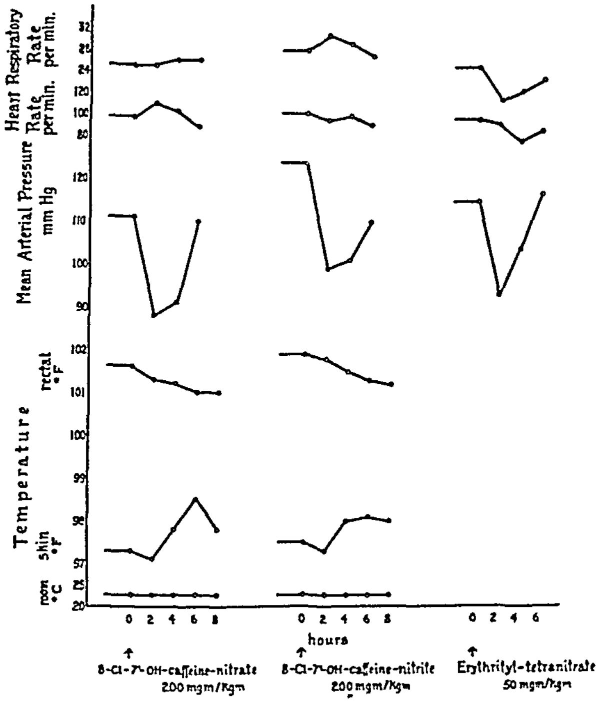


FIG. 6. AVERAGES OF OBSERVATIONS ON FIVE DOGS, EXCEPT THREE ONLY FOR ERYTHRITYL-TETRANITRATE[2]

*Toxic action of single doses.* In table 1 are shown the mortality ratios for these compounds following gastric administration in suspension in acacia solution to rats. The low toxicity is doubtless associated with slight solubility in water.

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[2] The erythrityl-tetranitrate used in this study was kindly supplied by Mercd & Co., Inc., Rahway, N. J.

The symptoms of poisoning included loss of normal posture, dyspnea, and cyanosis. Death occurred after a sudden attack of violent convulsions. Rigor mortis set in immediately. These observations suggest that the xanthine moiety of the molecule exerts the chief toxic action.

*Toxic action of repeated doses.* The gastric administration to rats of 250 mgm./kgm. per day of the chlorohydroxycaffeine nitrite and nitrate esters led to death within 6 to 8 days. Determination of blood iron (Wong's method) and oxygen capacity (method of Van Slyke and Neill) and spectroscopic examination gave no evidence of formation of methemoglobin.

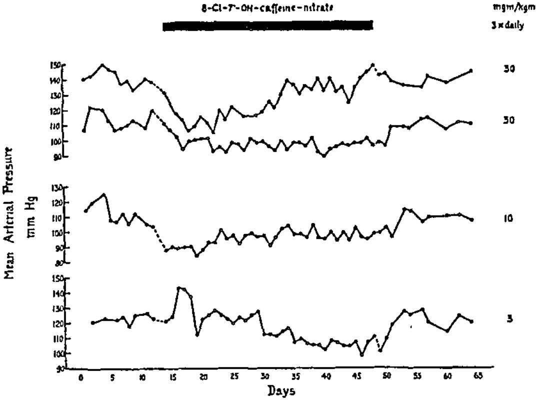

FIG 7. Mean femoral arterial pressure in two dogs receiving three times daily 30 mgm. per kgm., one dog receiving three times daily 10 mgm. per kgm., one dog receiving three times daily 3 mgm. per kgm., of 8-Cl-7'-OH-caffeine nitrate.

To 40 rats, chlorohydroxycaffeine nitrate ester was given by incorporating it in the food. The animals' weight and food intake were measured weekly, and drug intake calculated. The results are shown in figure 8. Of the ten rats at the dietary level of 0.2%, only four survived until the thirteenth day; these were then moribund and were killed. The average daily drug intake at this level was 171 mgm./kgm. Of the ten rats at the level of 0.1%, six had died by 49 days, and the rest survived, but growth was impaired. The average drug intake in this group was 65 mgm./kgm./day. At the 0.05% and 0.025% levels, food intake and growth were not distinguishably different from the controls. The average daily drug intake at these levels was 31 and 14 mgm./kgm., respectively. Specimens of heart, lung, liver, spleen, stomach, small intestine, kidney, and urinary bladder of all rats, except those dying during the night, were taken for histological examination. Dr. A. J. Miller, Professor of Pathology, found no histological changes attributable to the drug. At necropsy, the stomachs of the rats at the 0.2% level showed the mucosa to be edematous, but microscopic examination was negative.

To 4 apparently healthy male mongrel dogs, chlorohydroxycaffeine nitrate ester was administered orally in formaldehyde-treated gelatin capsules in single doses of 100–200 mgm. per kgm. Blood erythrocytes, hemoglobin as acid hematin, blood iron by Wong's method, blood oxygen capacity, renal clearance of creatinine, were determined before the administration of the drug, and at intervals during the administration. Microscopic examination of urinary sediment, and tests for albumin and sugar in the urine were made at intervals throughout the experiment. The results are shown in figure 9. Blood iron and oxygen capacity were determined only in Dog 3 on the sixteenth day, and in Dog 4 on the thirtieth day of administration. The values did not differ significantly from the normal. Examination of the urinary sediment and for sugar and albumin gave negative findings throughout. At the end of the experiment, the dogs were killled and submitted to necropsy, with microscopic examination of heart,

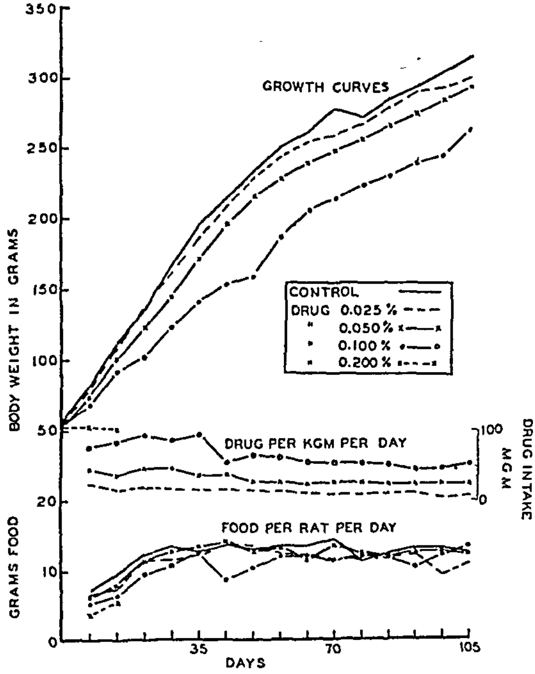

FIG. 8. Average values of body weight, food intake and drug intake of rats receiving 8-Cl-7′-OH-caffeine nitrate. At each concentration of the drug in the diet ten rats. Control group ten rats.

lung, liver, spleen, kidney, stomach, and small intestine. No pathological conditions attributable to the drug were found. The only positive findings of this experiment were in the behavior of the dogs. During the first 20 days of the experiment all of them vomited occasionally about two hours after the administration. In Dogs 1 and 2 these symptoms then subsided and were absent for the remaining time. In Dogs 3 and 4, vomiting continued and diarrhea appeared. In Dog 3 these effects stopped at the end of the first period of administration, and only occasional vomiting was seen after the daily dose had again been

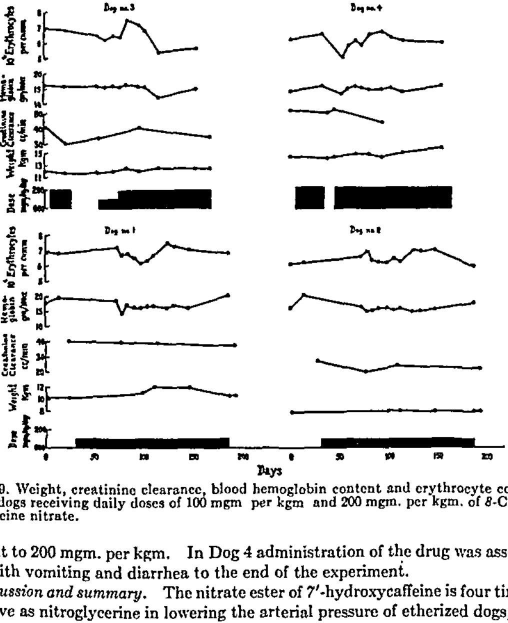

FIG. 9. Weight, creatinine clearance, blood hemoglobin content and erythrocyte count in four dogs receiving daily doses of 100 mgm per kgm and 200 mgm. per kgm. of 8-Cl-7'-OH-caffeine nitrate.

brought to 200 mgm. per kgm. In Dog 4 administration of the drug was associated with vomiting and diarrhea to the end of the experiment.

*Discussion and summary.* The nitrate ester of 7'-hydroxycaffeine is four times as active as nitroglycerine in lowering the arterial pressure of etherized dogs, on intravenous injection of equal weights, although nitroglycerine has three times the number of nitrate groups. The addition of an 8-chloro group reduces this activity but raises the fatal dose almost four times. The nitrate and nitrite of this chlorohydroxycaffeine exhibit activities characteristic of organic nitrates. They produce a fall in arterial pressure, vasodilation in the extremities and coronary circulation, a rise in skin temperature, and an increase in intracranial pressure. Under suitable circumstances, such as in the isolated heart and heart-lung preparation, it can be shown that larger doses will lead to a response like that to other xanthine derivatives, but in the whole animal the intense vasodepressor action interferes with the demonstration of such properties. In unanesthetized dogs chlorohydroxycaffeine nitrate, given three times daily in a dose of 10 mgm. per kgm., or possibly less, is capable of keeping arterial pressure below normal. There is evidence of development of tolerance at a larger dose.

The daily administration to rats of chlorohydroxycaffeine nitrate in doses of 170 mgm. per kgm. or more leads to the death of all animals. There is no evidence of toxic action at lower dosage levels except at doses which eventually kill some animals of the group. Dogs are less susceptible to the toxic action of repeated doses. As much as 200 mgm. per kgm. per day had no discernible damaging action, except locally in the alimentary tract.

*8*-chloro-*7′*-hydroxycaffeine nitrate is a potent vasodilator substance with prolonged action and low toxicity. It seems deserving of clinical trial.

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# MORPHOGENETIC ACTIONS OF VARIOUS STEROIDS IN THE CASTRATE MALE RAT

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In a previous communication we described the effect of various steroids on the morphogenetic actions of estradiol (1). Among the steroids studied in that paper several proved capable of inhibiting certain actions of estradiol, for instance, the testis and accessory sex organ involution which usually results from treatment with folliculoid compounds. It appeared particularly noteworthy that pregnenolone (*17*-ethyl-$\Delta^5$-androstene-*3*($\beta$)-ol-*20*-one) and progesterone markedly stimulate the seminal vesicles of mature rats in which the testes and accessory sex organs have undergone atrophy following estradiol treatment while they exert no such effect in the immature castrate. The question arose as to whether these steroids stimulate the seminal vesicles only in the presence of testicular tissue (which, in the case of estradiol-treated animals, may be atrophic) or whether their activity is dependent upon the maturity of the animals. The first possibility would suggest that certain steroids are gonadotropic in the male and cause the discharge of testoid substances from the testis. The second possibility should likewise be considered since it has been shown that immediately after puberty there occurs a marked increase in the sensitivity of the castrate rat to the testoid action of certain steroids (2).

In order to examine these possibilities and to obtain further evidence concerning the correlations between the chemical structure and the morphogenetic effects of various steroids, a number of experiments were carried out on immature and mature gonadectomized males whose organs were examined following treatment with a large variety of steroid compounds.

EXPERIMENTAL. Our first experiments were performed on immature male albino rats weighing 40–60 g. They received 2 daily subcutaneous injections of 0.1 cc. of peanut oil, each containing 5 mg. of the steroid to be examined. The latter was present in the oil as a fine crystalline suspension. Treatment was continued for 10 days, the animals being killed on the 11th day at which time the organs were carefully dissected and weighed after fixation in "Susa" solution.

Table 1 summarizes our findings. The full chemical name is used to describe the structure of each steroid, but the common name (in italics) is likewise mentioned. The melting point of the sample we used was always determined in our laboratory and is mentioned in the table as an indicator of the purity of our preparations. It may also help to identify compounds (especially isomerids) whose chemical structure is not as yet quite certain. The correct melting point of the most highly purified preparations described in the literature are recorded in brackets for comparative purposes. Six animals were used for each compound. In every case the average organ weight is given in the Table, the maximum spread being indicated in brackets.

TAB

*Morphogenetic actions of various steroids administered in high dosages to immature castrate rats*

| GROUP NO. | TREATMENT | M.P. | DOSAGE | INITIAL BODY WEIGHT | FINAL BODY WEIGHT | PITUITARY | ADRENAL | THYMUS | SEMINAL VESICLES | VENTRAL PROSTATE | MIDDLE PROSTATE | PREPUTIAL GLAND | COAGULATING GLAND | KIDNEY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | °C. | mg. | g. | g. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | g. |
| 1 | 17-methyl- $\Delta^4$-androstene-3-one-17($\alpha$)-ol<br>*Methyl testosterone* | 153<br>(163–164) | 10 | 49<br>(40–55) | 77<br>(67–98) | 3<br>(2–4) | 22<br>(21–26) | 59<br>(40–103) | 197<br>(162–222) | 132<br>(96–178) | 89<br>(82–91) | 45<br>(39–50) | 49<br>(40–60) | 0.895<br>(0.843–0.969) |
| 2 | 17-ethinyl- $\Delta^4$-androstene-3-one-17-ol<br>*Ethinyl testosterone* | 265–268<br>(270–272) | 10 | 46<br>(40–58) | 88<br>(80–100) | 3<br>(3–3.5) | 20<br>(16–22) | 151<br>(129–175) | 79<br>(62–91) | 55<br>(46–70) | 44<br>(36–49) | 46<br>(30–59) | 16<br>(9–21) | * |
| 3 | $\Delta^5$-androstene-3($\beta$)-ol-17-one<br>*Dehydro-isoandrosterone* | 137<br>(144–146) | 10 | 48<br>(40–60) | 93<br>(85–105) | 3.5<br>(3–4) | 22<br>(17–30) | 137<br>(84–200) | 64<br>(40–95) | 89<br>(74–100) | 56<br>(48–62) | 92<br>(76–107) | 21<br>(18–24) | 1.014<br>(0.870–1.205) |
| 4 | $\Delta^5$-androstene-3($\beta$)-17($\alpha$)-diol<br>*Androstenediol* | 182–183<br>(182–183) | 10 | 46<br>(40–58) | 86<br>(75–100) | 3<br>(2–4) | 18<br>(14–20) | 193<br>(168–233) | 16<br>(12–19) | 39<br>(27–48) | 21<br>(16–23) | 62<br>(55–72) | 6<br>(2–11) | * |
| 5 | 17-*iso*-heptyl-$\Delta^4$-androstene-3,25-dione<br>*Nor-cholestenedione* | 114–116<br>(114–116) | 10 | 48<br>(40–60) | 90<br>(80–110) | 4.3<br>(3.4–6.1) | 23<br>(20–26) | 164<br>(96–203) | 11<br>(9–13) | 8<br>(7.5–10) | 13<br>(7–19) | 21<br>(18–25) | 3<br>(2–4.5) | 0.946<br>(0.783–1.193) |
| 6 | 17-ethyl-$\Delta^4$-androstene-3,20-dione<br>*Progesterone* | 128<br>(128) | 10 | 48<br>(40–55) | 90<br>(80–100) | 4<br>(3.5–4.5) | 15<br>(10–22) | 157<br>(133–195) | 9<br>(8–10) | 52<br>(43–59) | 28<br>(22–39) | 36<br>(26–53) | 4<br>(3–4) | 0.946<br>(0.812–1.070) |
| 7 | 17-ethyl- $\Delta^5$-androstene-3($\beta$)-ol-20-one<br>*Pregnenolone* | 186<br>(185–187) | 10 | 47<br>(40–55) | 90<br>(75–107) | 5<br>(4–6) | 22<br>(18–26) | 246<br>(166–294) | 7<br>(6–7) | 23<br>(17–41) | 14<br>(7–19) | 29<br>(18–41) | 3<br>(3–4) | 0.979<br>(0.914–1.078) |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | *17-iso*-decyl-$\Delta^{5,22}$-androstadiene-*3*(β)-ol-acetate<br>*Stigmasterol acetate* | 168–169<br>(168–169) | 10 | 46<br>(40–55) | 91<br>(80–100) | 3<br>(3–4) | 25<br>(23–27) | 244<br>(183–326) | 7<br>(5–9) | 8<br>(7–9) | 11<br>(6–15) | 22<br>(17–28) | 2<br>(2–3) | * |
| 9 | *17*-ethyl-etiocholane-*3,20*-dione<br>*Pregnanedione* | 121<br>(120) | 10 | 47<br>(40–55) | 92<br>(85–110) | 5<br>(5–6) | 22<br>(16–31) | 252<br>(207–278) | 6<br>(5–7) | 8<br>(6–10) | 10<br>(8–11) | 16<br>(11–25) | 3<br>(2–4) | 1.062<br>(0.982–1.168) |
| 10 | *17*-ethyl-$\Delta^{4}$-androstene-*3,20*-dione-*21*,ol-acetate<br>*Desoxycorticosterone acetate* | 152<br>(158–160) | 10 | 40<br>(40–42) | 72<br>(70–75) | 3<br>(3–4) | 12<br>(10–13) | 94<br>(78–128) | 9†<br>(7–10) | 5<br>(4–7) | 11<br>(9–15) | 17<br>(10–20) | * | * |
| 11 | *17-iso*-octyl-$\Delta^{5}$-androstene-*3*(β)-ol<br>*Cholesterol* | 149<br>(150) | 10 | 45<br>(30–55) | 88<br>(60–105) | * | * | * | 8†<br>(6–9) | 17‡<br>(12–21) | | 22<br>(18–30) | * | * |
| 12 | *17-iso*-heptyl-$\Delta^{5}$-androstene-*3*(β)-ol-*25*-one<br>*Nor-cholestenolone* | 127–128<br>(125) | 10 | 48<br>(40–52) | 89<br>(80–95) | 3.6<br>(3.1–4) | 23.5<br>(20.6–29) | 181<br>(126–207) | 8<br>(7–9) | 9<br>(6–12.6) | 10<br>(7–13) | 15<br>(9–27) | 3<br>(2–5.3) | 0.802<br>(0.715–0.894) |
| 13 | *17*-ethyl-$\Delta^{5}$-androstene-3(β)-*21*-diol-*20*-one-*21*-acetate<br>*Acetoxy-pregnenolone* | 183–184<br>(183–184) | 10 | 45<br>(30–55) | 91<br>(70–105) | * | * | * | 8†<br>(6–10) | 15‡<br>(12–19) | | 19<br>(12–31) | * | * |
| 14 | *Peanut oil* | | | 49<br>(40–60) | 89<br>(80–105) | 5<br>(4–6) | 22<br>(18–25) | 228<br>(164–207) | 7<br>(5–8) | 7<br>(5–9) | 11<br>(6–14) | 14<br>(8–18) | 3<br>(2–4) | 1.026<br>(0.942–1.223) |

* Not weighed.

† In these cases the seminal vesicles were weighed together with the coagulating glands.

‡ In these cases the ventral and middle prostates were weighed conjointly.

The most striking facts which emerge from table 1 are that androstenediol (Cpd. 4) causes a slight, while progesterone (Cpd. 6) and desoxycorticosterone acetate (Cpd. 10) elicit a marked decrease in the size of the adrenals. These observations are in agreement with our previous findings in adult rats of both sexes. It will be recalled that the atrophy of the adrenals elicited by these steroids is limited to the cortical part of the gland.

The thymus is subnormal in most experimental groups with the exception of the animals receiving peanut oil (which acted as controls), stigmasterol acetate (Cpd. 8), pregnanedione (Cpd. 9) and pregnenolone (Cpd. 7). It will be noted that all of these compounds proved hormonally inactive except for pregnenolone which has a very slight prostate-stimulating effect. This observation is in agreement with the view expressed by Selye (3) according to which the production of thymus atrophy is a common property of the hormonally active steroids and is independent of the specific nature of their hormonal effects.

The seminal vesicles steadily decrease in size from compound 1 to compound 14, as the groups in the table were specifically arranged according to this property. It is doubtful whether the apparent trace of seminal vesicle enlargement seen in the progesterone (Cpd. 6) group should be considered significant, but compounds 7 to 14 are certainly devoid of any capacity for enlarging the seminal vesicle. This is particularly interesting as regards pregnenolone (Cpd. 7) because, in intact mature males in which testicular and seminal vesicle atrophy were induced by estradiol treatment, we (1) noted that pregnenolone possesses a definite stimulant seminal effect on the seminal vesicles.

The ventral and, to a lesser degree, the middle prostate are enlarged by progesterone and pregnenolone (Cpds. 6, 7) although these substances exert little or no effect on the seminal vesicles. This adds further support to the view that the various male accessory sex organs may respond selectively to testoid compounds. This is also shown by the example of androstenediol (Cpd. 4) which stimulates the preputial glands proportionately much more intensely than the other accessory sex organs. The response of the coagulating glands, on the other hand, runs approximately parallel to that of the seminal vesicles, while the kidneys evidence no very clear-cut "pro-renal" action in these short term experiments.

*A second experimental series* was designed to study the morphogenetic actions of lower doses of the most active steroids enumerated in table 1 and also of some compounds which were available only in comparatively small amounts and hence could not be given at the dose level of 10 mg. a day. Testosterone, which is presumably the physiological testicular hormone, was administered at two different low dose levels for comparative purposes. Group 14 of Table I, which received peanut oil only, may serve as a control for this series as well, since the experiments of tables 1 and 2 were performed under exactly identical conditions except for the dosage used.

The figures in table 2 reveal no significant change in the weight of the pituitary, but they indicate a marked decrease in adrenal size in the group treated with Kendall's Compound "E" (Cpd. 10). This is in agreement with the concept of compensatory organ atrophy (4) according to which hormones cause atrophy of the gland by which they are normally produced if they are administered in excessively high dosages. With regard to the effect of the steroids on the accessory sex organs it is noteworthy that the two folliculoid compounds of this series (Cpds. 5, 7) cause definite enlargement of the seminal vesicles. This however, must not be taken as an indication of a true testoid effect since the enlargement is known to be due merely to fibro-muscular development in the wall of the seminal vesicles.

The definite prostatic enlargement caused by Kendall's Compound "E" (Cpd. 10) is unaccompanied by a simultaneous increase in the size of the seminal vesicles and in this respect the action of the above adrenal compound resembles that of progesterone and pregnenolone. The comparatively high preputial gland stimulating activity of androstenediol (Cpd. 8), which had been mentioned in connection with the previous experimental series, is also quite obvious in this group in which the compound was used at a lower dose level. It will be noted that with the only exception of methyl testosterone (Cpd. 1) the preputial glands in the androstenediol group are larger than in any other group recorded in table 2 although the seminal vesicles and prostates were not considerably enlarged in this group.

In this series the pro-renal action was not marked in the case of any compound because of the short period of treatment. Yet it is noteworthy that the animals receiving androsterone possess the largest average renal weight.

*The third series of experiments* was performed on adult castrate rats in order to determine whether the morphogenetic actions of the steroids would be altered by the age of the animal. The experiments were performed in exactly the same manner as in series 1 and 2, except that the average weight of the animals was 157–158 g. at the beginning of the experiment. In most cases the steroids were administered at the dose of 10 mg. per day except in the case of androsterone of which we could not secure sufficiently large quantities and in the case of the two folliculoid compounds (Cpds. 9, 11) which are toxic if given in excessively high amounts. These latter three compounds were administered in daily doses of 2 mg. It will also be noted that in this series a number of organs were examined which had not been weighed in the previous two experiments.

The largest final body weight was observed in the androsterone (Cpd. 5) series but the difference between these and the controls is not significant. On the other hand both folliculoid compounds (Cpds. 9, 11) caused pronounced inhibition of somatic growth in accordance with previous observations on this subject. The pituitary was slightly enlarged in the estradiol (Cpd. 9) series but showed no significant change in the other groups. There appears to have been slight thyroid atrophy in the testosterone and androsterone (Cpds. 1, 5) groups, but otherwise the thyroid weights did not deviate significantly from the normal.

The adrenals are enlarged in the groups treated with folliculoids (9, 11) and atrophic following administration of desoxycorticosterone acetate or progesterone (Cpds. 15, 14). The fact that folliculoids cause adrenal atrophy in immature and hypertrophy in mature animals is in agreement with previous observations (5).

The seminal vesicles were definitely enlarged by compounds 1 to 6 although

## TABLE 2

*Morphogenetic actions of various steroids administered in comparatively low dosages to immature castrate rats*

| GROUP NO. | TREATMENT | M.P. | DOSAGE | INITIAL BODY WEIGHT | FINAL BODY WEIGHT | PITUITARY | ADRENAL | THYMUS | SEMINAL VESICLES | VENTRAL PROSTATE | MIDDLE PROSTATE | PREPUTIAL GLAND | COAGULATING GLAND | KIDNEY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | °C. | mg. | g. | g. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | g. |
| 1 | *17*-methyl- $\Delta^4$-androstene-*3*-one-*17* (α)-ol<br>*Methyl testosterone* | 153<br>(163–164) | 2 | 49<br>(40–60) | 93<br>(83–101) | 4<br>(3–4) | 25<br>(22–28) | 121<br>(80–163) | 226<br>(196–257) | 145<br>(121–171) | 100<br>(96–100) | 63<br>(53–80) | 51<br>(33–76) | 1.036<br>(0.952–1.127) |
| 2 | *17*-ethinyl- $\Delta^4$-androstene-*3*-one-*17*-ol<br>*Ethinyl testosterone* | 265–268<br>(270–272) | 2 | 48<br>(40–60) | 92<br>(80–110) | 4<br>(3–4) | 17<br>(13–21) | 217<br>(177–260) | 43<br>(36–51) | 55<br>(31–64) | 38<br>(32–46) | 29<br>(15–56) | 11<br>(9–13) | 0.965<br>(0.853–1.024) |
| 3 | Androstane-*3* (α)-ol-*17*-one<br>*Androsterone* | 177–178 | 2 | 47<br>(45–50) | 85<br>(75–95) | 4<br>(3–4) | 19<br>(16–24) | 194<br>(127–235) | 25<br>(18–34) | 100<br>(88–110) | 63<br>(42–74) | 36<br>(28–47) | 11<br>(8–15) | 1.094<br>(0.936–1.273) |
| 4 | $\Delta^4$-androstene-*3*-one-*17*(α)-ol<br>*Testosterone* | 154<br>(154–154.5) | 0.20 | 48<br>(40–55) | 85<br>(70–100) | 4<br>(3–4) | 20<br>(16–25) | 156<br>(134–186) | 25<br>(15–32) | 49<br>(43–57) | 26<br>(19–32) | 17<br>(12–20) | 8<br>(5–11) | 0.920<br>(0.728–1.081) |
| 5 | *17*-ethinyl-$\Delta^{1,3,5}$-estratriene-*3,17* (α)-diol<br>*Ethinyl estradiol* | 138–139<br>(145–146) | 2 | 48<br>(40–55) | 76<br>(65–85) | 4.9<br>(4.2–5.8) | 21<br>(19–23) | 98<br>(75–112) | 24<br>(19–26) | 9<br>(6–11) | 16<br>(9–20) | 20<br>(17–26) | 7<br>(4–9) | 0.822<br>(0.735–0.866) |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | $\Delta^4$-androstene-*3*-one-*17*(α)-ol<br>*Testosterone* | 154<br>(154–154.5) | 0.05 | 48<br>(40–55) | 85<br>(65–100) | 4<br>(3–4) | 20<br>(14–26) | 184<br>(125–220) | 19<br>(15–26) | 39<br>(33–56) | 21<br>(16–26) | 16<br>(10–24) | 5<br>(4–7) | 0.890<br>(0.669–1.061) |
| 7 | $\Delta^{1,3,5}$-estra-triene-*3,17*(α)-diol<br>*α-Estradiol* | 176<br>(176–177) | 2 | 48<br>(40–50) | 74<br>(65–80) | 4<br>(3–5) | 16<br>(15–19) | 92<br>(68–108) | 19<br>(17–23) | 8<br>(7–11) | 11<br>(9–12) | 17<br>(13–22) | 5<br>(4–6) | 0.851<br>(0.732–0.945) |
| 8 | $\Delta^5$-androstene-*3*(β)-*17*(α)-diol<br>*Androstenediol* | 182–183<br>(182–183) | 2 | 47<br>(40–55) | 90<br>(85–100) | 3<br>(2–4) | 17<br>(13–20) | 256<br>(213–342) | 14<br>(11–18) | 36<br>(29–44) | 18<br>(14–25) | 47<br>(31–78) | 4<br>(3–6) | 1.051<br>(0.950–1.241) |
| 9 | $\Delta^5$-androstene-*3*(β)-ol-*17*-one<br>*Dehydro-iso-androsterone* | 137<br>(144–146) | 2 | 47<br>(40–55) | 88<br>(70–110) | 4<br>(3–5) | 16<br>(14–19) | 228<br>(165–282) | 7<br>(6–9) | 37<br>(29–42) | 14<br>(12–17) | 26<br>(17–43) | 4<br>(2–4) | 0.956<br>(0.772–1.159) |
| 10 | *17*-ethyl- $\Delta^4$-androstene-*3,11,20*-trione-*17,21*-diol<br>*Kendall's compound "E"* | 215–216<br>(215–218) | 1 | 47<br>(40–55) | 74<br>(60–85) | 4<br>(3–5) | 14<br>(13–15) | 106<br>(74–152) | 7<br>(5–9) | 24<br>(22–28) | 11<br>(8–13) | 14<br>(11–17) | 3<br>(2–4) | 0.913<br>(0.900–0.930) |
| 11 | *17*-ethyl- $\Delta^5$-androstene-*3*(β)-ol-*20*-one<br>*Pregnenolone* | 186<br>(185–187) | 2 | 48<br>(42–54) | 88<br>(75–100) | 4<br>(4–5) | 25<br>(22–28) | 241<br>(194–340) | 6<br>(5–7) | 19<br>(10–25) | 11<br>(10–12) | 19<br>(15–24) | 3<br>(2–3) | 0.957<br>(0.847–1.022) |

TABLE 3

*Morphogenetic actions of various steroids in mature castrate rats*

| GROUP NO. | TREATMENT | M.P. | DOSAGE | INITIAL BODY WT. | FINAL BODY WT. | PIT. | THYROID | ADRENAL | THYMUS | SEMINAL VES. | VENTRAL PROSTATE | MIDDLE PROSTATE | PREP. GLANDS | COAG. GLANDS | COWPER GLANDS | KIDNEY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | °C. | *mg./day* | *g.* | *g.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *g.* |
| 1 | Δ⁴-androstene-*3*-one-*17*(α)-ol<br>*Testosterone* | 154<br>(154–154.5) | 10 | 158<br>(140–180) | 187<br>(160–210) | 5.8<br>(5–7) | 13<br>(11–15) | 30<br>(25–37) | 112<br>(88–150) | 1124<br>(877–1278) | 391<br>(301–535) | 274<br>(226–362) | 117<br>(92–136) | 210<br>(171–322) | 68<br>(59–75) | 1.575<br>(1.266–1.858) |
| 2 | *17*-methyl-Δ⁴-androstene-*3*-one-*17*(α)-ol<br>*Methyl testosterone* | 153<br>(163–164) | 10 | 157<br>(135–175) | 182<br>(130–215) | 7.1<br>(6.5–8) | 15.7<br>(11–20) | 33<br>(29–38) | 162<br>(87–192) | 906<br>(717–1160) | 387<br>(345–458) | 284<br>(218–336) | 125<br>(99–171) | 180<br>(143–247) | 71<br>(56–92) | 1.663<br>(1.362–2.039) |
| 3 | Δ⁵-androstene-*3*(β)-ol-*17*-one<br>*Dehydro-iso-androsterone* | 137<br>(144–146) | 10 | 158<br>(143–180) | 173<br>(155–195) | 6.4<br>(5–7.5) | 15<br>(10–19) | 28.5<br>(23–34) | 149<br>(46–209) | 404<br>(207–682) | 198<br>(118–356) | 138<br>(106–193) | 97<br>(67–141) | 77<br>(54–129) | 38<br>(32–53) | 1.527<br>(1.431–1.708) |
| 4 | *17*-ethinyl-Δ⁴-androstene-*3*-one-*17*-ol<br>*Ethinyl testosterone* | 265–268<br>(270–272) | 10 | 157<br>(142–170) | 184<br>(156–210) | 7.1<br>(6–9.3) | 15<br>(11–17) | 31<br>(22–35) | 268<br>(140–392) | 206<br>(113–238) | 142<br>(92–196) | 101<br>(67–125) | 60<br>(41–90) | 49<br>(31–58) | 25<br>(17–35) | 1.520<br>(1.330–1.760) |
| 5 | Androstane-*3*(α)-ol-*17*-one<br>*Androsterone* | 177–178<br>(180–181) | 2 | 158<br>(140–175) | 192<br>(170–210) | 7.4<br>(7–8) | 12.6<br>(9–15.4) | 28<br>(21–29.6) | 332<br>(223–511) | 110<br>(27–167) | 158<br>(126–193) | 160<br>(78–202) | 63<br>(44–96) | 30<br>(10–40) | 23<br>(14–32) | 1.703<br>(1.500–1.871) |
| 6 | Δ⁵-androstene-*3*(β)-*17*(α)-diol<br>*Androstenediol* | 182–183<br>(182–183) | 10 | 158<br>(140–175) | 186<br>(165–195) | 7.4<br>(6.7–8.2) | 17<br>(15–20) | 32<br>(26–38) | 339<br>(293–370) | 69<br>(60–92) | 62<br>(55–68) | 70<br>(59–87) | 112<br>(77–199) | 22<br>(17–33) | 20<br>(13–25) | 1.652<br>(1.472–1.850) |
| 7 | *17*-ethyl-Δ⁵-androstene-*3*(β)-*21*-diol-*20*-one-*21* acetate<br>*Acetoxypregnenolone* | 183–184<br>(183–184) | 10 | 158<br>(147–175) | 185<br>(160–210) | 7.2<br>(5.8–9.0) | 14<br>(11–16) | 30<br>(26–32) | 286<br>(155–382) | 65<br>(46–85) | 54<br>(14–96) | 50<br>(41–58) | 53<br>(40–94) | 22<br>(17–24) | 18<br>(13–26) | 1.681<br>(1.503–1.802) |
| 8 | *17*-*iso*-heptyl-Δ⁵-androstene-*3*(β)-ol-*25*-one<br>*Nor-cholestenolone* | 127–128<br>(125) | 10 | 158<br>(140–175) | 188<br>(170–215) | 7.4<br>(5–9) | 16<br>(13–19) | 32<br>(28–38) | 336<br>(240–571) | 65<br>(35–105) | 36<br>(27–48) | 45<br>(35–58) | 56<br>(36–88) | 23<br>(12–42) | 16<br>(13–20) | 1.510<br>(1.258–1.682) |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | $\Delta^{1,3,5}$-estratriene-*3, 17(α)*-diol<br>*α-Estradiol* | 176<br>(176–177) | 2 | 157<br>(140–180) | 134<br>(120–145) | 8.4<br>(6.5–10.5) | 15<br>(13–17) | 46<br>(31–60) | 75-<br>(54–107) | 65<br>(51–80) | 33<br>(21–44) | 54<br>(44–67) | 34<br>(25–43) | 17<br>(15–21) | 11.5<br>(9–16) | 1.306<br>(1.209–1.500) |
| 10 | *17-iso*-octyl-$\Delta^{5}$-androstene-*3(β)*-ol<br>*Cholesterol* | 149<br>(150) | 10 | 157<br>(145–180) | 188<br>(175–205) | 7.7<br>(6.7–10.3) | 17<br>(16–19) | 29<br>(26–33) | 343<br>(220–449) | 64<br>(32–95) | 37<br>(20–47) | 51<br>(40–70) | 62<br>(40–90) | 22<br>(11–36) | 16<br>(7–24) | 1.397<br>(1.310–1.521) |
| 11 | *17*-ethinyl-$\Delta^{1,3,5}$-estratriene-*3,17(α)*-diol<br>*Ethinyl estradiol* | 138–139<br>(145–146) | 2 | 157<br>(140–175) | 132<br>(125–160) | 7.4<br>(5 5–9.4) | 14.4<br>(12–19) | 56<br>(41–80) | 53.5<br>(36–102) | 57<br>(40–93) | 33<br>(22–59) | 45<br>(31–91) | 47<br>(36–63) | 15<br>(9–24) | 10<br>(8–12) | 1.302<br>(1.151–1.413) |
| 12 | *17*-ethyl-$\Delta^{5}$-androstene-*3(β)*-ol-*20*-one<br>*Pregnenolone* | 188–189<br>(185–187) | 10 | 158<br>(146–170) | 189<br>(170–200) | 7.4<br>(5.6–9) | 14.7<br>(11–18) | 28<br>(24–33) | 284<br>(190–407) | 55<br>(39–77) | 34<br>(17–60) | 46<br>(33–59) | 74<br>(52–99) | 14 6<br>(11–20) | 11.8<br>(8–16) | 1.525<br>(1.254–1.724) |
| 13 | *Peanut oil*—0 2 ml. per day | | | 158<br>(150–165) | 189<br>(175–200) | 7.3<br>(6 6–8) | 16<br>(12–19) | 31<br>(24–37) | 345<br>(267–405) | 50<br>(39–64) | 28<br>(21–31) | 34<br>(29–39) | 48<br>(41–62) | 12<br>(8.5–17) | 10.8<br>(7–13) | 1.497<br>(1.347–1.600) |
| 14 | *17*-ethyl-$\Delta^{4}$-androstene-*3,20* dione<br>*Progesterone* | 128<br>(128) | 10 | 158<br>(140–185) | 166<br>(135 200) | 6.9<br>(5.4–8.4) | 15<br>(11–19) | 26.5<br>(20–33) | 246<br>(153–330) | 45<br>(23–61) | 62<br>(40–96) | 60<br>(49–78) | 51<br>(26–69) | 14<br>(11–17) | 13<br>(7–20) | 1 394<br>(1.112–1.555) |
| 15 | *17*-ethyl-$\Delta^{4}$-androstene-*3,20*-dione-*21*-ol-acetate<br>*Desoxycorticosterone acetate* | 152<br>(158–160) | 10 | 158<br>(150–175) | 178<br>(160–193) | 7 3<br>(6.4–8) | 15<br>(13–18) | 17<br>(14–19) | 203<br>(120–354) | 41<br>(29–55) | 27<br>(12–43) | 32<br>(24–44) | 41<br>(29–48) | 12<br>(9–15) | 11<br>(5–18) | 1.714<br>(1 584–1.855) |

the action of androstenediol (Cpd. 6) was rather doubtful at this dose level. The other steroids were entirely inert. This lack of activity in these adult castrates is again of particular interest in the case of pregnenolone and progesterone (Cpds. 12, 14) since, as we mentioned above, both these steroids stimulate the seminal vesicles in the presence of testicular tissue in intact adult rats. The same is true of androstenediol, whose activity was rather doubtful in the present series of castrates, while it exerted a very pronounced stimulant effect on the seminal vesicles in the intact rat (1). Thus our experiments prove conclusively that the responsiveness of the seminal vesicles to steroids such as progesterone and pregnenolone is not dependent upon the age of the animal, but upon the presence of testis tissue.

In this series again the prostate, and especially its ventral lobe, was stimulated most markedly by androsterone, which compound differed in its effect from most other steroids examined in that it made the absolute weight of the ventral prostate larger than that of the seminal vesicles. It will be noted that the other compounds cause much more pronounced seminal vesicle stimulation with the exception of those substances (*e.g.*, progesterone) which have no effect on the seminal vesicles of castrates. As in all previously mentioned experiments the preputial glands responded comparatively most intensely to androstenediol, which appears to be a relatively specific stimulator of these organs. The coagulating and Cowper gland weights ran approximately parallel with those of the seminal vesicles and hence deserve no special comment.

The pro-renal effect was fairly evident in the case of the testoids and desoxycorticosterone actate, while the folliculoids, estradiol and ethinyl estradiol (Cpds. 9, 11), exerted an anti-renal action.

It is noteworthy that the male accessory sex organs of the animals treated with desoxycorticosterone acetate, as recorded in tables 1 and 3, were actually subnormal in size. Although this involution below the normal castrate level is not very pronounced it may indicate that the compensatory atrophy of the adrenal cortex caused by this compound interferes with the normal testoid production of the adrenal cortex. In other words, it might eliminate an accessory endogenous source of testoids which is still present after gonadectomy in the untreated organism. In any case none of our experiments support the view expressed by others (6) who believed this compound to be "androgenic" or testoid.

From the point of view of the correlations which exist between the chemical structure and the pharmacological activity of the steroids, the following facts appear to emerge from the above experimental series. The presence of an acetylated $C_{21}$ hydroxy group is detrimental for the prostate-stimulating activity, as shown by the examples of progesterone and pregnenolone on the one side, and desoxycorticosterone acetate and acetoxypregnenolone on the other. The latter two compounds differ from the former only in that they possess an additional acetoxy group at $C_{21}$ and in both these cases the prostate stimulating activity vanishes following introduction of this group. It will be remembered that the introduction of a $C_{21}$ acetoxy group causes a similar inhibition of the vagina and uterus stimulating effects of progesterone and pregnenolone (3).

The results reported in this communication are in agreement with the previously expressed view (3) according to which etiocholanes, *17*-alkyl substituted etiocholanes, and all steroids having a *C17* side-chain of 7 or more carbon atoms, are devoid of hormonal activity.

The finding that Kendall's Compound "E" possesses prostate-stimulating activity is of some interest as it represents the most highly oxygenated compound which has so far been shown to be endowed with some type of testoid activity. While this shows that the introduction of as many as five oxygen atoms does not necessarily destroy the ability of a steroid to stimulate the male accessory sex organs, yet the compounds examined support the view (3) that the greatest testoid potency is exhibited by steroids oxygenated only at the two extreme poles of the molecule.

Finally it is noteworthy that the introduction of a 2-carbon-atom side-chain at $C_{17}$ does not endow an estratriene with testoid activity as shown by the example of ethinyl-estradiol. It will be remembered that most of the similarly substituted androstane derivatives are endowed with some measure of testoid potency.

## SUMMARY

The morphogenetic action of a number of steroid compounds has been examined under comparable laboratory conditions in immature and adult castrate male rats. The most outstanding results of these investigations were the following:

Certain steroids stimulate the male accessory sex organs in a relatively selective manner. Thus testosterone stimulates the seminal vesicles, androsterone the prostate, and androstenediol the preputial glands comparatively more than the other accessory sex organs.

Kendall's Compound "E" is endowed with a definite though not very intense prostate-stimulating activity.

The presence of testicular tissue is indispensable for the seminal-vesicle-stimulating action of certain steroids such as progesterone and pregnenolone. Other compounds (*e.g.*, androstenediol) exert some seminal-vesicle-stimulating action even in the castrate but their activity is greatly increased in the presence of testicular tissue.

Contrary to previous claims desoxycorticosterone acetate is not only devoid of any testoid action but actually tends to decrease the size of the accessory sex organs below the normal castrate level. This, it is assumed, is due perhaps to the compensatory adrenal atrophy with its resulting inhibition of endogenous testoid production by the adrenal cortex.

The correlations between the chemical structure and the pharmacological activity of the steroids used in these experiments have been discussed.

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# THE ASCORBIC ACID—DEHYDROASCORBIC ACID SYSTEM IN THE SYNTHESIS AND INACTIVATION OF SYMPATHOMIMETIC AMINES[1]

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Recent work on the elimination of sympathomimetic amines by the body has shown some of them to be excreted as such by the kidneys (1, 2, 3). Others not normally excreted as such we have found to be enzymically deaminated by amine oxidase (4, 5). However, some of these compounds which have not been found to be inactivated enzymically are not totally excreted as such or conjugated.

As a result of investigating other possible mechanisms for the inactivation of the amount of amphetamine not excreted as such we found vitamin C capable of deaminating the compound *in vitro* and also of decreasing the excretion of the amine as such when the vitamin was given to dogs (6). The purpose of this investigation has been to determine to what extent the configuration of the molecule influences the reactions of sympathomimetic amines with the ascorbic acid—dehydroascorbic acid system.

EXPERIMENTAL. The method used for measuring the *in vitro* deamination of sympathomimetic amines was as follows: Duplicate tubes containing 50 mgm. ascorbic acid and from 50 to 100 mgm. of an amine either as its sulfate or hydrochloride were buffered at pH 7.0 by 30 cc. M/16 phosphate buffer and oxygenated for 18 to 24 hours at 37°C. These were set up together with duplicate controls containing only one or the other of the two components, ascorbic acid or amine. At the end of the period of oxygenation the solutions were made basic and the $NH_3$ distilled at 80°C. into a known volume of standardized HCl. The excess acid was titrated with standardized NaOH against methyl orange as an indicator and the ammonia calculated.

RESULTS. Tables 1 and 2 summarize the results of experiments using 9 compounds representing six alterations in the basic phenylethylamine structure which are known to modify enzymic inactivation of and physiological response to the compounds. The average figures in the tables represent from 2 to 6 determinations on each compound at the concentration given.

*The deamination of a primary amine* by the ascorbic acid—dehydroascorbic acid system when the compound contains no aromatic or aliphatic hydroxyl groups was not primarily dependent on the position of the amino group, as may be seen in table 1. This is in contradistinction to the action of amine oxidase which is capable of deaminating only those compounds having an amino group on a terminal carbon atom (4). However, from the data it would seem that those compounds having an amino group on the side chain in a position *beta*

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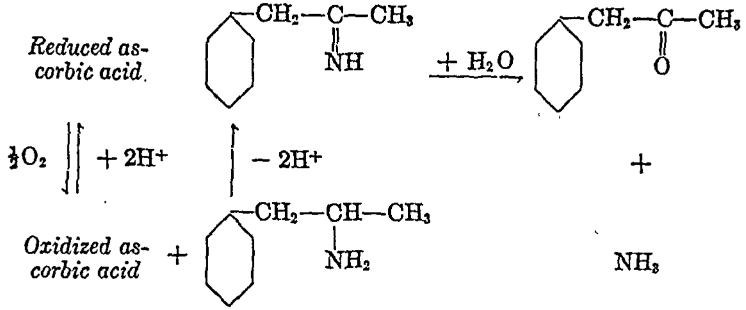
to the phenyl nucleus are more easily deaminated than when it is three carbon atoms removed from the ring. The reactions involved in the deamination of these primary amines are probably as follows (*equation 1*):



To supplement the evidence for deamination and ketone formation we have determined the presence of the ketone by its hydrazone following reaction with *2-4* dinitrophenylhydrazine. To 1 cc. of the oxygenated solution of the amine and ascorbic acid was added 1 cc. of a saturated solution of *2-4* dinitrophenylhydrazine in normal HCl. This was allowed to stand at room temperature for half an hour during which time a fine yellow precipitate formed. Then 2 cc. of normal NaOH were added to the precipitated hydrazone whereupon the dark red-brown color due to the hydrazone solution developed and remained over night. The test may be used for aldehydes and ketones.

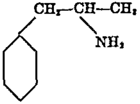
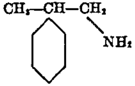
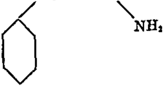
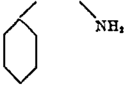
The existence of *an hydroxyl group on the side chain,* as in propadrine, (table 2) markedly decreased the deamination of the compound (9 to 11.9%) as compared to its homolog amphetamine, table 1 (31 to 54.1%), which does not have the hydroxyl group. The presence of a secondary amino group as in ephedrine instead of the primary amino group, propadrine, did not alter significantly the action of the hydroxyl group in retarding deamination.

The effect of the dipole moment of the aliphatic hydroxyl group is opposite in sign and of greater magnitude than the induced effect of the primary or secondary amine on the molecular moment of the compound. Since these two groups are only one carbon atom removed, in the case of propadrine and ephedrine, it is to be expected that the reactivity of the primary or secondary amino group would be depressed somewhat by the hydroxyl group. As further evidence for this suppression of amine reactivity by a *beta* aliphatic hydroxyl group may be cited the coupling reactions of two such compounds as amphetamine and propadrine with *p*-nitrobenzenediazonium chloride (7). In both instances the wave-length for maximal light absorption and the character of the absorption spectrum of the color of the coupled compounds in butanol are the same. However, for equimolar concentrations of the two amines the absorption coefficient for amphetamine at its wave-length of maximal light absorption is significantly greater than for propadrine, which has the aliphatic hydroxyl group. In the two instances coupling with the diazonium compound is through the amine.

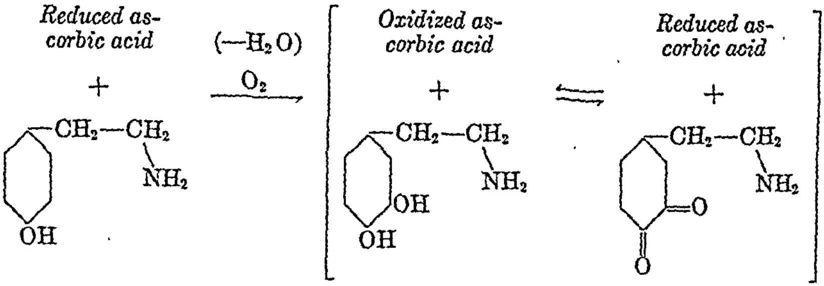
*The tertiary amine,* β-phenylisopropyldimethylamine, was resistant to deamination by this system. As a result of work not yet published we have found that other tertiary amines are refractory to deamination by amine oxidase. Primary and secondary amines in general are capable of undergoing much the same reactions in deamination, there being formed ammonia or methylamine. However, in the case of the tertiary amines it is impossible for the formation of the imino group to take place as illustrated in equation 1, since two hydrogen atoms on the same *C—N* linkage are not available for reduction of the dehydroascorbic acid.

TABLE 1

*The relation of molecular configuration to the deamination of sympathomimetic amines by the ascorbic acid-dehydroascorbic acid system*

| AMINE FORMULA | AMINE | ASCORBIC ACID | THEORETICAL RECOVERY OF $NH_3$ | AMMONIA RECOVERED (AV.) | PER CENT $NH_3$ RECOVERED (AV.) |
|---|---|---|---|---|---|
| | mgm. | mgm. | mgm. | mgm. | |
| Amphetamine<br> | $SO_4$ | | | | |
| | 50 | 50 | 4.6 | 2.49 | 54.1 |
| | 75 | 60 | 6.9 | 2.13 | 31.0 |
| | 100 | 50 | 9.2 | 3.21 | 34.9 |
| β-phenpropylamine<br> | $SO_4$ | | | | |
| | 50 | 50 | 4.6 | 2.46 | 53.5 |
| | 75 | 50 | 6.9 | 2.89 | 42.1 |
| | 100 | 50 | 9.2 | 3.75 | 44.9 |
| γ-phenpropylamine<br> | $SO_4$ | | | | |
| | 50 | 50 | 4.6 | .56 | 12.3 |
| | 75 | 50 | 6.9 | 2.15 | 31.2 |
| | 100 | 50 | 9.2 | 2.10 | 22.8 |
| Phenethylamine<br> | HCl | | | | |
| | 50 | 50 | 5.4 | 2.29 | 42.5 |
| | 75 | 50 | 8.1 | 2.83 | 35.0 |
| | 100 | 50 | 10.8 | 3.25 | 30.0 |

*Sympathomimetic amines having an aromatic hydroxyl group in the para position* were not deaminated under these conditions by ascorbic acid even when the compounds contained a primary amino group (table 2). These observations may be explained by the following equation for the reactions as we believe them to occur in the case of these compounds (*equation 2*).



According to equation 2, the equivalent of one molecule of oxygen is shared to oxidize the ascorbic acid to dehydroascorbic acid and the *p*-hydroxyphenethylamine (tyramine) to its orthodihydroxy homolog. The dehydroascorbic acid oxidizes the catechol nucleus to the *ortho* quinonoid state and is itself reduced. This gives rise to an oxidation-reduction equilibrium which does not permit deamination to take place. Obviously, sympathomimetic amines having a catechol nucleus would not be deaminated by ascorbic acid since they would fit into the above equilibrium.

The following evidence is in support of this equation:

*1.* That there is formed an *ortho* dihydroxy nucleus in the course of the reaction can be shown by Richter's test for adrenalin and its homologs (8). This test depends on the presence of a *3-4* dihydroxyphenyl nucleus and an ethylamine or isopropylamine side chain, and can be used in the presence of ascorbic acid. This last is a point of advantage over most of the tests, as $FeCl_3$. This test depends on the adsorption of the *ortho* dihydroxy compound from solution on $Al(OH)_3$ at pH 8 and elution with $Na_2HPO_4$. Iodine in KI solution is added to the phosphate solution which is buffered at pH 5.2 with acetate buffer, and the excess iodine is oxidized with sodium thiosulfate leaving the pink color of the iodine complex formed with the adrenalin-like compound.

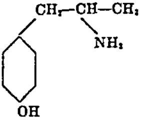
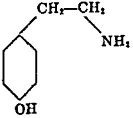
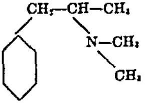
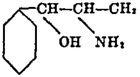
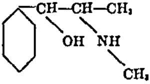
*2.* The formation of the *ortho* dihydroxy nucleus is dependent on the presence of the ascorbic-dehydroascorbic acid system for the oxidation and is not due to autoxidation of the initial compound. Tyramine can be vigorously oxygenated for 24 hours at the end of which time the above test for the catechol nucleus is negative unless ascorbic acid was initially present, also.

*3.* Depending on the *p*-hydroxy derivative used, when air is bubbled through a solution of the amine and ascorbic acid at 37°C. there is formed fairly soon an orange, orange-pink or orange-brown color. This is in contrast to a faint straw color which becomes apparent after vitamin C and amphetamine or a similar compound are oxygenated for about 18 hours. In view of the equations this may be taken as presumptive evidence for quinone formation.

*4.* This color formation does not occur when tyramine is oxygenated under similar circumstances but without the presence of ascorbic acid in the solution. Thus the ascorbic acid is necessary for the reaction to go to quinone formation.

TABLE 2

*The relation of molecular configuration to the deamination of sympathomimetic amines by the ascorbic acid-dehydroascorbic acid system*

| AMINE FORMULA | AMINE | ASCORBIC ACID | THEORETICAL RECOVERY OF $NH_3$ | AMMONIA RECOVERED (AV.) | PER CENT $NH_3$ RECOVERED (AV.) |
|---|---|---|---|---|---|
| | *mgm.* | *mgm.* | *mgm.* | *mgm.* | |
| Paredrine<br> | HBr | | | | |
| | 50 | 50 | 3.7 | 00 | 00 |
| | 75 | 50 | 5.2 | 00 | 00 |
| | 100 | 50 | 7.4 | 00 | 00 |
| Tyramine<br> | HCl | | | | |
| | 50 | 50 | 4.9 | 00 | 00 |
| | 75 | 50 | 7.4 | 00 | 00 |
| | 100 | 50 | 9.8 | 00 | 00 |
| β-phenisopropyldimethylamine<br> | HCl | | $(CH_3)_2NH$ | | |
| | 50 | 50 | 11.7 | 00 | 00 |
| | 75 | 50 | 17.6 | 00 | 00 |
| | 100 | 50 | 23.5 | 00 | 00 |
| Propadrine<br> | HCl | | | | |
| | 50 | 50 | 4.6 | .42 | 9.0 |
| | 75 | 50 | 6.8 | .81 | 11.9 |
| | 100 | 50 | 9.1 | .86 | 9.4 |
| Ephedrine<br> | $SO_4$ | | $CH_3NH_2$ | $CH_3NH_2$ | $CH_3NH_2$ |
| | 50 | 50 | 5.1 | .54 | 10.6 |
| | 75 | 50 | 7.6 | .81 | 10.7 |
| | 100 | 50 | 10.2 | .61 | 6.0 |

*5.* That the color formed is due to some labile system capable of being easily reduced may be shown by the addition of a trace of sodium bisulfite to the colored solution. Thereupon, the color quickly disappears.

Discussion. These experiments serve to emphasize another factor in the inactivation and also synthesis of the sympathomimetic amines. The significance of ascorbic acid in the deamination of primary and secondary sympathomimetic amines having no aromatic hydroxyl group lies in the fact that hereby one may explain why those compounds not inactivated by amine oxidase are not entirely excreted as such or conjugated. Both the vitamin C and amine oxidase systems deaminate primary and secondary (sympathomimetic) amines when the amino group is on the terminal carbon atom but, of the two, only the ascorbic acid system will deaminate the isopropylamine homologs. Lending support to this view are the following *in vivo* experiments. We have shown that by giving an hepatotoxin, carbon tetrachloride, in sufficient amounts to inhibit secondarily or partially destroy amine oxidase, β-phenylpropylamine and γ-phenylpropylamine can be caused to be excreted as such; whereas they are ordinarily deaminated by amine oxidase and are not normally excreted as such when given in 10 to 25 mgm. doses (5). On the other hand $CCl_4$ also causes a depletion of the vitamin C content of the liver and other organs (10). Therefore since β-phenylisopropylamine (amphetamine) is deaminated by ascorbic acid it is understandable that by giving carbon tetrachloride one can cause all instead of only part of a given dose of the amine to be excreted even though amphetamine is not deaminated by amine oxidase (9). Conversely, giving dogs injections of 200 mgm. of ascorbic acid per day cuts down the excretion of amphetamine to about one-third of the amount normally excreted by the animals (6).

The *in vitro* oxidation of the *para* hydroxy phenyl sympathomimetic amines to their corresponding *ortho* dihydroxy homologs with the subsequent inhibition of destruction of the new compound, suggests the possibility of a new rôle for ascorbic acid. It may well be that it brings about a step in the synthesis of adrenalin from its monohydroxy precursors. This hypothesis is attractive because of the abundance of ascorbic acid in the body, the fact that this step does take place *in vitro* and the lack of positive evidence that there is present in the body a phenol oxidase capable of bringing about this transformation. Schemes for the synthesis of adrenalin in the body have simply assumed a phenol oxidase to be present in mammalian tissues since it is widely distributed in plants and some lower animals. So far there has been prepared from mammalian tissues no phenol oxidase capable of oxidizing compounds of the nature of tyramine or paredrine to the corresponding amine having a catechol nucleus.

It has been known for some time that a relatively large amount of ascorbic acid is stored in the adrenal glands. Heard and Welch (11) have admirably shown that the presence of vitamin C aids in the stabilization of adrenalin during its storage in the adrenal gland. Our experiments directly bear out their observations concerning the inhibitory effect of vitamin C on the inactivation of adrenalin homologs, once they have been formed.

## SUMMARY

By means of an *in vitro* method, it has been shown that there is a definite relationship between molecular configuration and the reactions of sympatho-

mimetic amines with the ascorbic acid-dehydroascorbic acid system. It was observed that:

1. Compounds having no hydroxyl group on the ring and having a primary amino group on the side chain were deaminated with a recovery of 30 to 54.1% of the theoretical yield of ammonia.
2. An hydroxyl group on the side chain *beta* to the primary amino group decreased the deamination of the compounds to about 10% of the theoretical recovery (propadrine 9 to 11.9%).
3. A secondary methyl group in addition to the aliphatic hydroxyl radical did not materially affect the deamination (ephedrine 6 to 10.7%) as compared to the corresponding primary amine (propadrine).
4. A tertiary amine on the side chain precluded the possibility of deamination by this system.
5. Where an hydroxyl group existed in the *para* position on the aromatic nucleus the compound was oxidized to the *ortho* dihydroxy compound. Deamination was inhibited since the catechol nucleus and its corresponding ortho quinone entered into oxidation-reduction equilibrium with the ascorbic acid-dehydroascorbic acid system.

The possibility that ascorbic acid serves (*1*) in the deamination of certain sympathomimetic amines not inactivated by amine oxidase (*2*) in the synthesis of the ortho dihydroxy from the para hydroxy adrenalin precursors and (*3*) in the stabilization of adrenalin in the body has been proposed and discussed.

The author wishes to acknowledge his appreciation of the criticism of this paper by Dr. Walter J. Meek.

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# IN VITRO STUDIES OF SULFONAMIDE ACTION ON ORGANISMS OF THE BRUCELLA GROUP AND THE COUNTERACTING EFFECT OF PARA-AMINOBENZOIC ACID[1]

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The effects of various sulfonamide compounds on *Brucella melitensis*, as determined by the Warburg manometric method, have been previously reported (1). Of the compounds then available for clinical use, sulfathiazole was found to have the most marked bacteriostatic effect on this organism. The present studies were undertaken to compare the bacteriostatic action of sulfathiazole, sulfadiazine and sulfaguanidine on various brucella strains, and to determine the ability of *p*-aminobenzoic acid to inhibit the action of sulfathiazole.

MATERIAL AND METHODS. For these experiments four strains of *Brucella suis*, one strain of *Brucella melitensis*, and two strains of *Brucella abortus*, were used. Two of the suis strains (L and M) were isolated from human sources, one (ABF 36) from an animal source, and one (47) was a stock strain. The melitensis strain (428) was a stock culture which received three mouse passages before being used. The abortus strains, which did not require $CO_2$ for growth, were obtained from the Animal Disease Station, Beltsville, Md.

After the organisms had grown for 18 to 20 hours on bacto-tryptose (Difco) agar slants, the growth was washed off with sterile physiologic saline and the density of the suspension adjusted by means of the photronreflectometer so that 0.1 cc. (size of inoculum) contained about 5 million organisms.

Bacto-tryptose (Difco) broth was used as culture medium. Both the sulfonamide drugs (sulfathiazole, sulfadiazine and sulfaguanidine) and *p*-aminobenzoic acid were dissolved in broth, and made up to the desired concentrations by dilution with broth. These broths were sterilized by autoclaving at 15 pounds pressure for 15 minutes.

The bacteriostatic action of these drugs and the counteracting effect of *p*-aminobenzoic acid were determined by means of the photronreflectometer (2). After 24, 48, and 72 hours' incubation at 37°C., samples were withdrawn from the culture tubes and the growth determined from the density readings. At the end of each experiment cultures were made on blood agar slants from the drug-free and drug-containing broths to insure the absence of contamination and viability of the organisms.

RESULTS. *Experiment I: A comparison of the bacteriostatic activity of sulfathiazole, sulfadiazine, and sulfaguanidine on 7 brucella strains.* In this experiment, sulfathiazole and sulfaguanidine were used in concentrations of 2.5, 5, 10, and 25 mg.%. Sulfadiazine was used in concentrations of 2.5, 5, and 10 mg.%. Drug-free broth cultures were used as controls. The broths (volume 5 cc.) were inoculated with 0.1 cc. (about 5 million organisms) of saline suspensions of 18 to 20 hour cultures of the various brucella strains. The results of this experiment are summarized in table 1 and figures 1 and 2 illustrate experiments on two of the strains studied.

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[1] This work was supported by the James A. Greene Research Fund.

It may be seen from table 1 that sulfathiazole and sulfadiazine had almost the same bacteriostatic effect. The bacteriostatic action of sulfaguanidine was more variable and in some instances much less than that of the two other drugs. Very small or no differences in the activity of sulfathiazole and sulfadiazine in increasing concentrations were observed but with sulfaguanidine significant increases in bacteriostatic action were frequently observed as the drug concentration rose. It is worthy of comment that the *Brucella abortus* strains studied, on which the bacteriostatic effect of sulfathiazole and sulfadiazine was most pronounced, possess extremely low virulence, whereas the virulence of the other strains can be considered moderate or marked.

*Experiment II: The ability of p-aminobenzoic acid to counteract the bacteriostatic activity of sulfathiazole.* For this experiment *Brucella suis* (strain ABF 36) was used. The inoculum was the same as in the preceding experiments. Sulfathiazole was used in concentrations of 1.0, 2.5, 5, 10, and 25 mg.%, with and without 0.1 mg.% and 1.0 mg.% of *p*-aminobenzoic acid. Drug-free broths and broths containing 0.1 mg.% and 1.0 mg.% of *p*-aminobenzoic acid were used as controls. Growth was determined by means of the photronreflectometer as in the preceding experiments. The results of this experiment are given in figure 3.

TABLE 1

*Percentage inhibition of growth after 72 hours' incubation at 37°C. of various brucella strains by sulfathiazole, sulfaguanidine, and sulfadiazine*

| | DRUG CONCENTRATIONS (MGM. PER CENT) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sulfathiazole | | | | Sulfaguanidine | | | | Sulfadiazine | | |
| | Brucella strains | | | | | | | | | | |
| | 2.5 | 5 | 10 | 25 | 2.5 | 5 | 10 | 25 | 2.5 | 5 | 10 |
| Br. suis (L)..... | 48.7 | 48.7 | 48 7 | 48.7 | 40.5 | 44.6 | 48.7 | 48.7 | 50.8 | 47.7 | 49.2 |
| Br. suis (M)... | 46 3 | 50.0 | 50.0 | 55.3 | 40.0 | 47.4 | 49.6 | 49.5 | 52.1 | 50.0 | 51.6 |
| Br. suis (47)... | 61.0 | 66.3 | 63.2 | 65.6 | 49.8 | 52.8 | 52.5 | 59.5 | 60.4 | 62.4 | 62.6 |
| Br. suis (ABF 36)...... | 50.5 | 50 5 | 54.2 | 52.6 | | 48.4 | 50.0 | 50.5 | 52.1 | 47.9 | 48.4 |
| Br. melitensis... | 31.4 | 36.2 | 42.9 | 36.7 | 3.8 | 5.2 | 15.7 | 20.5 | 31.0 | 31.4 | 40.5 |
| Br. abortus (S). | 73.8 | 77.4 | 76.9 | 79.0 | 60.5 | 60.0 | 66.2 | 71.8 | 74.4 | 75.9 | 77.4 |
| Br. abortus (S 1).......... | 82.6 | 81.7 | 81.1 | 82.7 | 16.2 | 37.7 | 55.5 | 70.2 | 77.5 | 74.5 | 82.6 |

As was found in the preceding experiments, there are very slight differences in growth with increasing sulfathiazole concentrations above 1 mg.%. It is apparent from figure 3 that 0.1 mg.% of *p*-aminobenzoic acid can partially counteract the inhibitory action of sulfathiazole. To a slight degree the effect of 0.1 mg.% of *p*-aminobenzoic acid tends to vary inversely with the concentration of sulfathiazole. In a concentration of 1 mg.%, *p*-aminobenzoic acid almost or completely counteracts the inhibitory action of sulfathiazole except in combination with a sulfathiazole concentration of 25 mg.%, at which a significant though moderate sulfathiazole inhibition persists.

*Experiment III: Persistence of growth inhibition by sulfathiazole and its counteraction by p-aminobenzoic acid.* In this experiment after 48 hours' incubation

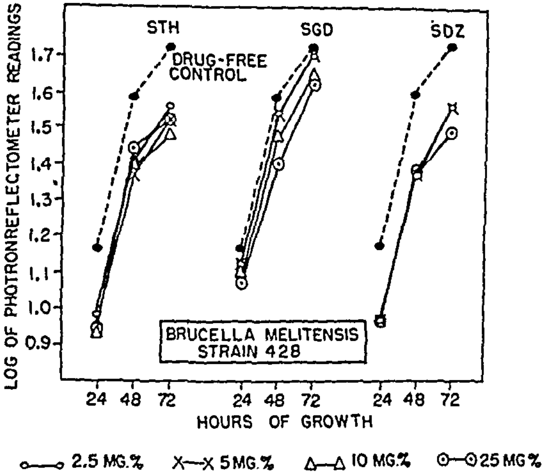

FIG. 1. TYPICAL EXPERIMENT ILLUSTRATING THE BACTERIOSTATIC ACTION OF SULFATHIAZOLE, SULFADIAZINE AND SULFAGUANIDINE ON BRUCELLA MELITENSIS (STRAIN 428)

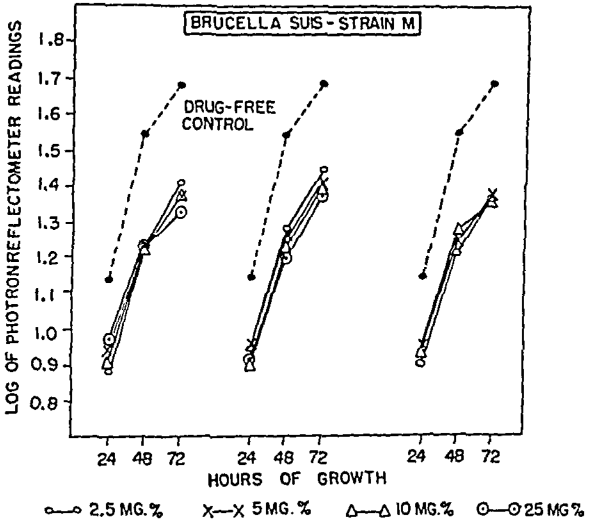

FIG. 2. TYPICAL EXPERIMENT ILLUSTRATING THE BACTERIOSTATIC ACTION OF SULFATHIAZOLE, SULFADIAZINE AND SULFAGUANIDINE ON BRUCELLA SUIS. (STRAIN M)

at 37°C., explants of 0.1 cc., each containing approximately 4 million organisms (*Brucella suis*, strain ABF 36) were made from the drug-free, and the sulfathiazole broth cultures to drug-free broth, and to drug-containing broths of the same concentrations, with and without 1 mg.% of *p*-aminobenzoic acid. Explants from the drug-free broth culture were also made to broth containing 0.1 cc. of each of the sulfathiazole broths in order to determine whether "carry over"

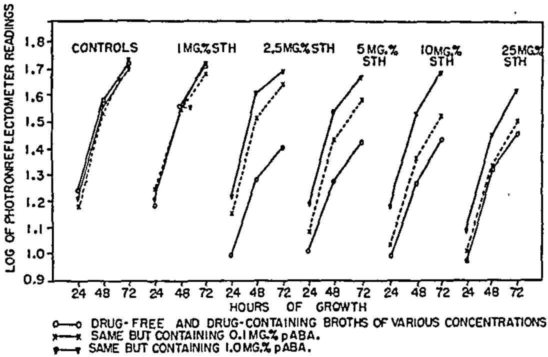

FIG. 3. ILLUSTRATING THE ABILITY OF *p*-AMINO-BENZOIC ACID TO INHIBIT SULFATHIAZOLE ACTION ON BRUCELLA SUIS. (STRAIN ABF36)

TABLE 2

*Growth in 48 hours of explants from drug-free and sulfathiazole broths*

| | DRUG-FREE BROTH | SULFATHIAZOLE CONCENTRATIONS (MGM. %) | | | | |
|---|---|---|---|---|---|---|
| | | *1* | *2.5* | *5.0* | *10.0* | *25.0* |
| Explants from drug-free broth to broth containing 0.1 cc. of each drug concentration | 38.75* | 36.0 | 31.0 | 29.75 | 28.75 | 29.75 |
| Explants from drug-free and drug-containing broths to drug-free broths | 38.75 | 16.75 | 8.25 | 6.25 | 4.5 | 4.0 |
| Explants from drug-containing broths to drug-containing broths of the same concentration | | 8.25 | 6.0 | 5.25 | 4.75 | 4.25 |
| Explants from drug-containing broths to drug-containing broths of the same concentration but containing 1 mg. per cent of para-aminobenzoic acid | | 40.5 | 34.25 | 34.5 | 32.25 | 22.0 |

* The figures are the readings of the photronreflectometer after 48 hours' incubation of the cultures at 37°C.

of drug in the explants could explain the continued inhibitory effect observed when explants were made from drug-containing broth to drug-free broth.

The results of this experiment are given in table 2. Comparison of the growth of explants from the drug-free broth to broths containing 0.1 cc. of each of the sulfathiazole concentrations (*i.e.*, the amount of drug "carried over" when explants were made from drug-containing broths) with that of explants made from drug-containing broths to drug-free broths, shows a marked difference, suggesting that the metabolic activities of the drug-exposed bacteria have been seriously impaired. Comparison of the growth of explants from drug-containing broths to drug-free and drug-containing broths of the same concentrations, shows a marked bacteriostatic effect, which, in sulfathiazole concentrations above 1 mg. %, is of similar magnitude. This bacteriostatic effect was markedly inhibited when 1 mg.% of *p*-aminobenzoic acid was added to the drug-containing broths. Since every culture was proved viable at the end of the experimental period, and since the explants were identical, this ability of *p*-aminobenzoic acid to counteract the inhibitory action of sulfathiazole emphasizes further the persistence of the sulfathiazole effect upon the bacteria after they have been transplanted to drug-free media, regardless of how this effect may be interpreted.

## SUMMARY

Comparison of the bacteriostatic action of sulfathiazole, sulfadiazine, and sulfaguanidine on 7 brucella strains has shown sulfathiazole and sulfadiazine to have almost the same effect. The bacteriostatic effect of sulfaguanidine is more variable, and frequently much less marked than that of the other drugs. Sulfathiazole and sulfadiazine, in increasing concentrations above 2.5 mg.%, exhibit very small or no differences in bacteriostatic action, whereas the bacteriostatic effect of sulfaguanidine frequently varies directly with the drug concentration.

*P*-aminobenzoic acid in concentrations of 0.1 and 1.0 mg.%, partially or almost completely inhibits the action of sulfathiazole.

Organisms exposed to the action of sulfathiazole for 48 hours, then explanted to drug-free broth, show a continued inhibition of growth, which is not due to "carry over" of sulfathiazole in the explants. This persisting inhibitory effect is counteracted by *p*-aminobenzoic acid.

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# EFFECT OF VARIOUS SULFONAMIDES, SULFONES, AND OTHER COMPOUNDS AGAINST EXPERIMENTAL INFLUENZA AND POLIOMYELITIS INFECTIONS IN WHITE MICE

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We have tested a number of sulfonamides, sulfones, and other compounds (principally heterocycles) for their effectiveness against two experimental virus infections in white mice. None of the drugs were effective in delaying or preventing the development of the disease, or in reducing the mortality from the infection. However, because a considerable number of compounds were tested, and because many of them were representative of large groups of related chemicals, the reporting of these negative results is considered to be of value.

MATERIAL AND METHODS. The viruses used were the PR8 strain of influenza A virus (supplied by Dr. F. L. Horsfall, Jr.) and the 18th mouse brain passage of the Armstrong strain of poliomyelitis virus (supplied by Dr. Max Theiler). Six to twelve Swiss mice weighing 12 to 20 grams each were used for each test. If a doubtful result was obtained, the test was repeated with a larger number of mice.

A fairly constant intake and blood concentration of drug over the entire period of therapy were insured by mixing the drug in known percentage with the food (1), which was made available to the mice 24 hours a day. The diet consisted of a balanced chick mash which had been sifted to reduce it to a homogeneous powder. Weighed amounts of powdered drug were added to the mash, which was then shaken in a cardboard container to distribute the drug evenly throughout. Sulfanilamide analyses of aliquots of such mixtures indicated that an even distribution of drug was obtained by these means. Mice fed well on this diet, consuming from 3.5 to 4.0 grams daily per 15 gram mouse. If any signs of toxicity were noted, the percentage of drug in the diet was reduced.

Certain drugs were given intraperitoneally, in which case they were dissolved or suspended in sterile physiological saline so that 0.25 or 0.5 cc. contained the dose to be given. Injections were made once daily for 6 to 8 days.

Twenty-four to 48 hours after the administration of drug was begun, the mice were inoculated with the infecting agent. Influenza A virus (PR8) was instilled intranasally under light ether anesthesia, in 0.05 cc. amounts of a $10^{-4}$ dilution (2 to 10 M.L.D.). The poliomyelitis virus (AV 18) was injected intracerebrally under ether anesthesia, the dose being 0.03 cc. of a $10^{-2}$ suspension of infected mouse brain. This amount of virus usually killed all the control mice.

The feeding or injection of drug was then continued for at least 5 days after inoculation, and the mice were observed for illness. In groups inoculated with influenza, mice found dead were autopsied and were considered to have died of the disease if more than one-half the total pulmonary volume was consolidated. On the 10th day after inoculation all surviving mice were chloroformed and autopsied, and the extent of pulmonary consolidation was recorded. In groups inoculated with poliomyelitis, the mice were observed for 21 days and were examined daily for signs of paralysis, which usually preceded death by 24 or 36 hours.

Blood level determinations of sulfonamide drugs were not considered necessary, because there was no question of comparing the relative effectiveness of partially effective drugs, and also since adequate blood levels of many of the drugs are known to be maintained by this method of feeding (1).

TABLE 1

*Compounds found ineffective against PR8 and AV18 viruses in white mice*

Sulfonamide derivatives

| SULFONAMIDE | PR8 DOSAGE AND ROUTE OF ADMINISTRATION | AV18 DOSAGE AND ROUTE OF ADMINISTRATION |
|---|---|---|
| *6* - Methoxy - *8* - aminoquinoline - *5* - azophenylsulfonamide di HCl* | 5 mgm. ip x 6 days | 5 mgm. ip x 6 days |
| *2*-Sulfanilamido-benzothiazole* | 3 mgm. ip x 6 days<br>1% po x 6 days | 3 mgm. ip x 6 days |
| Sulfanilamidoindazole-*5** | 2.5 mgm. ip x 6 days<br>0.5% po x 6 days | 2.5 mgm. ip x 6 days<br>0.5% po x 6 days |
| Sulfanilamidobenzotriazole-7* | 5 mgm. ip x 6 days<br>0.5% po x 6 days | 5 mgm. ip x 6 days |
| *4*(*N*-*2*,*5*-dimethyl pyrryl)-benzenesulfonamidothiazole-*2** | 5 mgm. ip x 6 days<br>2 mgm. ip x 6 days<br>1% po x 6 days | 5 mgm. ip x 6 days<br>2 mgm. ip x 6 days |
| *4*(*N*-*2*,*5*-dimethyl pyrryl)-benzene sulfonamidopyridine-*2** | 0.6 mgm. ip x 6 days<br>1.2 mgm. ip x 6 days<br>2.5 mgm. ip x 6 days<br>5 mgm. ip x 6 days | 1% po x 6 days<br>0.5% po x 6 days<br>0.1% po x 6 days<br>0.05% po x 6 days<br>0.6 mgm. ip x 6 days<br>1.25 mgm. ip x 6 days<br>2.5 mgm. ip x 6 days<br>5 mgm. ip x 6 days |
| *4*(*N*-*2*,*5*-dimethyl pyrryl) benzene sulfonamide* | 3 mgm. ip x 6 days<br>5 mgm. ip x 8 days<br>2 mgm. ip x 8 days<br>0.5% po x 6 days<br>1% po x 6 days | 5 mgm. ip x 8 days<br>2 mgm. ip x 8 days<br>0.2% po x 6 days<br>5 mgm. ip x 6 days |
| *N*¹-acetyl sulfanilamide[c] | 1% po x 6 days | |
| *N*¹-methyl sulfanilamide | 1% po x 2 days<br>0.2% po x 4 days | 0.2% po x 6 days |
| *N*¹-(*2*-hydroxyethyl) sulfanilamide[c] | 1% po x 6 days<br>2% po x 6 days<br>10 mgm. ip x 6 days | |
| Sulfanilylguanidine[c] | 1% x 6 days<br>2% x 6 days<br>5 mgm. ip x 6 days | 2% x 6 days |
| Sulfanilylmorphilide[c] | 1% po x 6 days | |
| *N*¹-sulfanilyl sulfanilic acid* | 1% po x 6 days | 5 mgm. ip x 6 days<br>1% po x 6 days |

TABLE 1—*Continued*

| SULFONAMIDE | PR8 DOSAGE AND ROUTE OF ADMINISTRATION | AV18 DOSAGE AND ROUTE OF ADMINISTRATION |
|---|---|---|
| Sulfadiazine[C] | 0.5% po x 6 days | 0.5% po x 6 days |
| Sulfamethylthiazole[C] | 1% po x 6 days | |
| Sulfamethyldiazine[C] | 1% po x 6 days | 1% po x 6 days |
| Na sulfanilyl arsanilate[†] | 3 mgm. ip x 6 days | 3 mgm. ip x 6 days |
| $N^1$-butyryl sulfanilamide[C] | 1% po x 5 days | 0.5% po x 6 days |
| $N^1$-furfuryl sulfanilamide[C] | 1% po x 4 days | |
| $N^1N^4$-bissulfanilyl acriflavine[C] | 5 mgm. ip x 6 days | 5 mgm. ip x 6 days |
| Quinine sulfathiazole bisulfate[L] | 1 mgm. ip x 6 days | 2 mgm. ip x 6 days |
| *2*-Sulfanilamido-*4*-phenylthiazole[W] | 5 mgm. ip x 5 days | 5 mgm. ip x 5 days |
| *3*-Chloro-*7*-methoxy-*9*(*4'*-sulfamidophenyl) aminoacridine[W] | 0.2% po x 5 days | |
| $N^1$-phenyl sulfanilamide | 1% po x 2 days<br>0.2% po x 4 days | 0.2% po x 6 days |
| $N^4$-sulfanilylsulfanilamide[C] | 5 mgm. ip x 6 days<br>1% po x 6 days | 5 mgm. ip x 6 days<br>1% po x 6 days |

[P] Supplied by Parke, Davis and Company.
[C] Supplied by Calco Chemical Company.
[†] Synthesized by *Dr. R. A. Shephard in the laboratory of Dr. E. K. Marshall, Jr.*
[L] Supplied by Eli Lilly Company.
[W] Supplied by Winthrop Chemical Company.

TABLE 2

*Compounds found ineffective against PR8 and AV18 viruses in white mice*

Sulfones

| SULFONE | PR8 DOSAGE AND ROUTE OF ADMINISTRATION | AV18 DOSAGE AND ROUTE OF ADMINISTRATION |
|---|---|---|
| 4 - Aminophenyl - 5′ - aminoquinolyl-8-sulfone* | 5 mgm. ip x 6 days | 5 mgm. ip x 6 days |
| 4,4′-Diaminodiphenyl sulfone-2-sulfonamide* | 3 mgm. ip x 6 days<br>2% po x 3 days<br>0.5% po x 6 days<br>3 mgm. ip x 6 days | 5 mgm. ip x 6 days<br>3 mgm. ip x 6 days |
| Na 4,4′-diaminodiphenyl sulfone-2-sulfonacetamide* | 1% po x 6 days<br>0.5% po x 6 days<br>5 mgm. ip x 6 days | 5 mgm. ip x 6 days<br>1% po x 6 days |
| Na 4,4′-diaminodiphenylsulfone N,N′-di (dextrose sulfonate)* | 20 mgm. ip x 6 days<br>0.5% po x 6 days<br>40 mgm. ip x 6 days | 20 mgm. ip x 6 days<br>0.5% po x 6 days<br>50 mgm. ip x 6 days |
| 4-Amino phenyl-5′-amino-2′pyridyl sulfone* | 0.2% po x 6 days<br>5 mgm. ip x 6 days | 1% po x 6 days |
| 4-Lauroyl amino-4′-amino diphenyl-sulfone* | 0.2% po x 6 days | |
| Diphenyl sulfone* | 0.2% po x 6 days | |
| 4,4′-Diethoxydiphenyl sulfoxide* | 0.2% po x 6 days | 0.2% po x 6 days |
| 4-Amino diphenyl sulfone* | 0.2% po x 6 days | |
| Disodium sulfone-acetyl-4,4′-diamino diphenyl sulfoxide* | 0.5% po x 6 days | 0.5% po x 6 days |
| Ethanolamine salt of 4-amino-4′-succinyl amino-diphenyl sulfone* | 0.4% po x 6 days | |
| 4 - Amino - 4′ - α - dimethylaminoacetylamino diphenyl sulfone methochloride* | 0.2% po x 6 days<br>0.1% po x 6 days | 0.1% po x 6 days |
| 2,4′-Diamino diphenyl sulfone-4-sulfonamide* | 0.2% po x 6 days | 0.2% po x 3 days |
| 4,4′-Dinitrodiphenyl sulfone* | 0.1% po x 6 days | 0.1% po x 6 days |
| 3,4′-Diaminodiphenyl sulfone* | 0.1% po x 6 days | |
| 2,4,4′-Triamino diphenyl sulfone* | 0.1% po x 6 days | |
| β-Aminoethyl-4-aminophenyl sulfone* | 0.2% po x 6 days | 0.2% po x 6 days |

TABLE 2—*Continued*

| SULFONE | PR8<br>DOSAGE AND ROUTE OF ADMINISTRATION | AV18<br>DOSAGE AND ROUTE OF ADMINISTRATION |
|---|---|---|
| *4,4'*-Diamino diphenyl sulfoxide* | 0.1% po x 6 days | 0.1% po x 6 days |
| *4,4',4"*-Triamino triphenyl sulfonium chloride* | 0.2% po x 6 days | |
| *2,4'*-Diamino-*5*-pyridyl phenyl sulfone* | 3 mgm. ip x 6 days | 0.5% po x 6 days<br>1% po x 6 days |
| Diacetyl diamino diphenyl sulfone[W] | 8 mgm. ip x 6 days | |
| *bis* (*N* [sodium naphthalene sulfonate-*2'*] carbamido-*N*-phenyl-*4*)sulfone[W] | 5 mgm. ip x 6 days | 0.5% po x 6 days |

* Supplied by Parke, Davis and Company.

[W] Supplied by Winthrop Chemical Company.

TABLE 3

*Compounds found ineffective against PR8 and AV18 viruses in white mice*

Quinoline derivatives

| QUINOLINE | PR8<br>DOSAGE AND ROUTE OF ADMINISTRATION | AV18<br>DOSAGE AND ROUTE OF ADMINISTRATION |
|---|---|---|
| Hydroxyethylapocupreine di HCl* | 1.5 mgm. ip x 6 days<br>1% po x 2 days<br>0.1% po x 4 days | 1.5 mgm. ip x 6 days |
| *4*-γ-Diethylamino-propylamino-*6*-methoxy-quinaldine *3* HCl* | 0.5% po x 6 days | |
| *2*-γ-Diethylamino-propylamino-*6*-methoxylepidine monohydrate* | 0.5% po x 6 days | |
| *4*-γ-*N*-morpholinopropyl-amino-*6*-methoxyquinaldine trihydrate* | 1% po x 3 days<br>0.2% po x 5 days<br>0.1% po x 6 days | |
| *6*-Methylquinoline-*2*-aldehyde* | 0.5% po x 6 days | |
| Quinoline *4*-aldehyde monohydrate* | 0.2% po x 6 days<br>5 mgm. ip x 6 days<br>0.5% po x 6 days | |
| *6*-Methyl quinoline-*8*-sulfonamide[C] | 1% po x 6 days<br>5 mgm. ip x 6 days | 5 mgm. ip x 6 days |

* Supplied by Parke, Davis and Company.

[C] Supplied by Calco Chemical Company.

TABLE 4

*Compounds found ineffective against PR8 and AV18 viruses in white mice*

Other compounds

| COMPOUND | PR8<br>DOSAGE AND ROUTE OF ADMINISTRATION | AV18<br>DOSAGE AND ROUTE OF ADMINISTRATION |
|---|---|---|
| *5*-Di-*n*-butylaminomethyl-*5*-methyl hydantoin* | 3 mgm. ip x 6 days<br>5 mgm. ip x 6 days<br>3 mgm. ip x 6 days | 3 mgm. ip x 6 days |
| *p*-tolamidine HCl* | 0.5% po x 6 days | 0.5% po x 6 days |
| *2*-*p*-Diethylaminostyryl benzoxazole methochloride* | 1% po x 6 days | 1% po x 6 days |
| *N*,*N′*-di-(*4*-hydroxyphenyl)-sebacamide* | 0.2% po x 6 days<br>5 mgm. ip x 6 days<br>0.5% po x 6 days | |
| *N*,*N′*-di-(*β*-aminoethyl)-sebacamide* | 0.2% po x 6 days | |
| Dithioacetyl-*p*-phenylenediamine* | 0.2% po x 6 days | |
| Theophylline* | 0.2% po x 6 days | |
| Aminophylline* | 0.2% po x 6 days | |
| *l*-Tetrahydropalmitine HCl* | 3 mgm. ip x 6 days | |
| Bis-*3*,*7*-(*p*-diethylaminobenzalimino) phenothiazine tri HCl* | 0.2% po x 6 days | |
| *p*-Diethylaminophenyl-*2*,*5*-dimethylpyrrole* | 1 mgm. ip x 7 days | 1 mgm. ip x 8 days |
| *2*,*5*-Diamino-*6*-hydroxy-pyrimidine sulfate* | 5 mgm. ip x 6 days | 5 mgm. ip x 6 days |
| Phenylazo-*α*, *α*-diamino pyridine HCl* | 3 mgm. ip x 4 days | |

* Supplied by Parke, Davis and Company.

Controls, fed the same drug mixtures but not infected with virus, were not included in the experiments. It was possible to differentiate between deaths due to toxicity and deaths due to virus infection, by the presence or absence of pulmonary consolidation in the case of influenza and paralysis in the case of poliomyelitis.

In tables 1, 2, 3, and 4 are listed the compounds tested against the two viruses, together with the dose in mgm. per mouse for intraperitoneal injection, or in percentage of drug in the mixture for oral feeding.

## BIBLIOGRAPHY

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# CERBERIN AND CERBEROSIDE, THE CARDIAC PRINCIPLES OF *CERBERA ODOLLAM*[1]

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*Cerbera odollam* is a tree of considerable height with dark-green, wedge-shaped leaves and yellowish-white flowers. It is a species of the family Apocynaceae. Its nuts when ripe are smooth on one side but rough on the other, weighing on the average 2.91 grams. When resting on the rough side they look like little turtles. They measure on the average 3.33 cm. in length, and 1.60 cm. in height. These nuts contain one kernel each, and are highly poisonous. The almond-shaped kernels, weighing on the average 2.12 grams each, are covered with a gray, hairy, firmly attached seed coat, and are composed of two cotyledons. The plant is indigenous to India, Dutch East Indies, Okinawa Island, and probably other islands of the Southwestern Pacific, particularly along the sea coast. The natives of Dutch East Indies call it Bintara in Malayan, or Bientaroh Gedeh in Sundanese language. In both British India and Dutch East Indies, the nuts have been employed for homicidal as well as suicidal purposes.

deVry (2) in 1864 reported his success in isolating a glycoside called cerberin from the expressed oil of the kernels. Subsequently, Oudemans (3), Ghanekar and Ayyar (4), and Kafuku and Hata (5) made an analysis of the fatty oil. Greshoff (6) claimed to have obtained a water-soluble glycoside, different from cerberin, and named it odollin. He did not, however, give his method of preparation, or physical constants and elementary analysis of his compound. Characterization of cerberin was chiefly accomplished by Plugge (7). Since *Cerbera odollam* belongs to the family Apocynaceae which gives rise to *Strophanthus* species, *Thevetia neriifolia*, *Nerium oleander*, and other digitalis-like drugs, it was natural for Plugge to show the systolic standstill of the frog's ventricle following the administration of cerberin or the crude extract (7, 8). At about the same time a dissertation was written by Zotos (9), but his work was severely criticised by Plugge (7). Recently Matsubara (10) reinvestigated the chemistry of cerberin, and proposed a structural formula.

Our interest in *Cerbera odollam* was to isolate cerberin and other active substances which might be present in the kernels of the nuts, and to conduct various pharmacologic experiments with the pure principles. Through the untiring effort of our Mr. Austin H. Brown, two shipments of the nuts, correctly identified by the Indian Forestry Department, were received from the same party in Bombay, India, one in 1938 and the other in 1940. The appearance and other features of the nuts answered the descriptions of the introductory paragraph.

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[1] Read in part at the New Orleans meeting of the Federation of American Societies for Experimental Biology, March, 1940 (1).

Briefly, the first batch of the nuts yielded 62% by weight of an ether soluble fatty oil, from which cerberin separated out. The latter when pure conformed to the empirical formula $C_{29}H_{46}O_8$. Cerberin could also be isolated from the defatted, powdered kernels by extraction with ethanol.

The second lot of the nuts was first percolated with petroleum ether. No cerberin settled out. The alcoholic extract of the defatted kernels first gave rise to sucrose, which was easily removed by filtration, and then to a bitter substance which could be purified by treatment with anhydrous aluminum oxide. To our surprise, this bitter principle was not the same as cerberin. Analytical results suggest the formula $C_{41}H_{70}O_{20}$. The name cerberoside is provisionally assigned with the hope that its relationship to cerberin may be somewhat similar to that between *K*-strophanthoside and *K*-strophanthin-$\beta$ (11). No explanation is as yet available regarding the difference in our findings except that many of the kernels in the first lot were almost black in contrast with the uniformly gray color in the second lot. Whether or not enzymatic action had taken place in the first lot is a question remaining to be investigated.

CHEMICAL PART. A total of 5.66 kgm. of kernels of *Cerbera odollam* was ground and percolated with ether. After the removal of ether by evaporation, the yellow fatty oil weighed 3.5 kgm., or 62% of the original material. On standing, a heavy, greasy sediment appeared which was separated by decantation. A portion of the sediment was almost insoluble in cold ethyl acetate, and tasted bitter. The residue separated by filtration was, however, soluble in warm ethyl acetate, and from this solution crops of crystals deposited. These proved to be cerberin. Purification was effected by repeated crystallization from warm ethyl acetate and decoloration with animal charcoal. The final product was obtained by recrystallization from methanol with the addition of water, drop by drop.

The defatted kernels were further pulverized and percolated with ethyl alcohol. The alcoholic extract was evaporated under reduced pressure, and the syrupy residue was diluted with water. The whole was extracted with chloroform several times. After the removal of chloroform by evaporation the dry resinous material was dissolved in absolute ethyl alcohol and from this solution an additional quantity of cerberin was recovered. Final purification was carried out in the same manner as above, namely, from methanol. Since it was aimed to prepare cerberin of the highest purity, no effort was made to improve the yield. The combined amount of pure cerberin from both the oil and the marc was only 5.15 grams.

Cerberin crystallizes in clusters of prisms. It softens at 201–205.5°C. and melts at 207.5–208.5°C. (corrected). Plugge (7) reported the melting point of 191–192°C.; and Matsubara (10), that of 191–193°C. In our preliminary communication (1), it was given as 202–202.5°C., but by further recrystallization this was raised to 207.5–208.5°C. It is levorotatory, having a specific rotation of $[\alpha]_D^{27}$ −46.9° in 95% ethyl alcohol. It is soluble in methanol, ethanol, isopropyl alcohol, pyridine, and chloroform, sparingly soluble in water, ethyl acetate, and acetone, and very slightly soluble in ether and benzene. It gives a positive test with sodium nitroprusside or Tollens' reagent. With concentrated sulfuric acid it produces a lemon yellow color turning to wine red with green fluorescence. It reacts with *m*-dinitro-benzene to form a violet color changing to indigo blue—a sensitive test for various cardiac glycosides described by Raymond (12). It develops an emerald green color with the Liebermann-Burchard reagent. The Keller-Killiani test was negative. Cerberin upon hydrolysis with dilute sulfuric acid in a sealed tube, reduces Benedict's solution.

Elementary analyses were made by Dr. Ing. A. Schoeller, Berlin-Schmargendorf (before World War II), and Dr. Carl Tiedcke, New York City, independently of each other. The results are concordant and suggestive of the empirical formula $C_{29}H_{46}O_8$ (calculated C = 66.62, H = 8.88; found with the specimen from the oil C = 66.48, 66 50, 66.73, 66.65, H = 8.71, 8.74, 8.77, 8.86; found with the specimen from the marc C = 66.37, 66 53, H = 8.77, 8.82). *It crystallizes with one-half molecule of water (for* $C_{29}H_{46}O_8 \cdot 1/2H_2O$, *calculated* $H_2O$ 1.72; found 1.75). The formula proposed by Plugge (7) was $C_{27}H_{40}O_8$, and that by Matsubara (10), $C_{29}H_{44}O_8$.

The second batch of the nuts of *Cerbera odollam* arrived as kernels weighing 21.3 kgm. They were ground, and this time percolated with gasoline. After the removal of the solvent by vacuum distillation, an orange-colored oil was recovered amounting to about 66% of the original weight. There was very little sediment from this oil, and no cerberin appeared. The defatted material was next percolated with ethyl ether. Upon evaporation of the percolate, only a small quantity of a waxy substance was obtained—insufficient for characterization. The marc was finally percolated with ethyl alcohol, and the combined extracts were distilled under diminished pressure. A repetition of the same procedure as employed in the first batch of the nuts with a small portion of the residue, at this stage, failed to yield any cerberin.

The main body of the alcoholic extract was evaporated under reduced pressure to complete dryness and pulverized. The powder was taken up in a minimum volume of absolute alcohol and fractionally precipitated by anhydrous ether. The precipitates were dissolved in absolute methanol. On standing over night the middle fractions deposited colorless crystals which were water-soluble and sweet to taste. When recrystallized from methanol, the substance melted at 186–186.5°C. (corrected) with decomposition. The yield of the pure material was 28.5 grams. The sweet taste, the melting point, and the specific rotation, $[\alpha]_D^{30} + 66.7°$, already suggest the possibility of sucrose. Analytical data prove that it is sucrose (calculated for $C_{12}H_{22}O_{11}$, C = 42.08, H = 6.48; found C = 41.95, 42.20, H = 6.09, 6.22).

The filtrates and non-crystalline precipitates from fractionation were combined and again evaporated to dryness. The powder became darker but possessed definite cardiac activity in frogs. A chromatographic experiment was tried by passing a 3% solution of the powder in absolute alcohol through a column (29 cm. long and 1.8 cm. in diameter) of anhydrous aluminum oxide according to Brockmann. No single band appeared, and the brown color spread over the whole column. It was soon found that aluminum oxide adsorbed the active principle along with the colored impurities and that it held the colored impurities more firmly than the other substance. This facilitated separation and purification. When the column was pushed out and treated with a sufficient amount of water, the solution, after filtering under suction, gave rise to a white, shining deposit on standing—bitter to taste and active on the frog's heart. The entire quantity of the dark brown powder, originally obtained from alcoholic extracts, was then triturated with anhydrous aluminum oxide in the proportion of 1 part of the oxide and 3 parts of the powder. A thin paste was made with water, and the whole filtered. A copious precipitate settled out from the filtrate on standing. This was separated by centrifuging, since it was difficult to filter. Second and third crops of the fine precipitate were obtained on further evaporation and standing. The final product was repeatedly crystallized from methanol-water mixture, or ethyl alcohol diluted with ethyl acetate. The yield of the pure material was 57 grams.

The substance, active on the frog's heart but apparently not identical with cerberin, crystallizes sometimes in rosettes, but frequently in fine plates of various shapes which occasionally form clusters, from an alcohol-ethyl acetate mixture. It shrinks at 186°C. and melts at 187.5–188.5°C. (corrected). Its specific rotation is $[\alpha]_D^{28}$ $-48°$. Its solubility in various solvents is similar to that of cerberin except that it is insoluble in chloroform and more soluble in water. Its color displays with various reagents are also closely comparable to those exhibited by cerberin. Upon hydrolysis with dilute sulfuric acid under pressure, a reducing component, presumably sugar, can be demonstrated with Benedict's solution. It is thus proposed to name the compound cerberoside, showing its relationship to cerberin and anticipating the possibility of its having the same aglycone.

Analysis made by Dr. Carl Tiedcke indicates the probable formula $C_{41}H_{70}O_{20}$ (calculated C = 55.75, H = 7.99; found C = 55.41, 55.80, H = 7.82, 7.89). This is of course provisional and subject to revision when more chemical data are available. No solvent of crystallization is present as revealed by heating to constant weight.

Pharmacological part. For animal experiments, stock solutions of 0.1% cerberin in 38% alcohol by volume, and 0.2% cerberoside in 19% alcohol, were suitable. From these dilutions were made as desired.

When adequate doses of either cerberin or cerberoside were injected into the frog's lymph sac, typical systolic arrest of the ventricle could be easily observed at the end of an hour. By perfusion into the inferior vena cava of 9 large Leopard frogs, according to the method of Howell and Cooke (13), cerberin in the concentrations of 1:250,000 and 1:125,000, induced systolic standstill within 40 minutes, preceded by slowing of the heart rate and later *A-V* block. A concentration of 1:500,000 produced a decrease of diastole and sinus rhythm, and appearance of premature beats.

Five similar perfusion experiments were carried out with cerberoside in frogs. A 1:20,000 solution caused stoppage of the ventricle in about an hour. A 1:50,000 solution greatly reduced the diastole, diminished the heart rate, and brought about several series of premature beats, but did not entirely stop the ventricular contraction at the end of an hour. A 1:100,000 concentration resulted in an initial augmentation of contraction and acceleration of heart rate, followed by bradycardia. The decrease of diastole was also obvious to the observer.

In etherized cats, sufficient amounts of cerberin or cerberoside, rapidly injected intravenously, caused a marked rise of blood pressure, arrhythmia, and sudden circulatory collapse—a picture common to all digitalis-like principles. When a 1:100,000 solution of cerberin was injected by the femoral vein at the rate of 1 cc. per minute into a series of 3 cats, anesthetized by ether, characteristic changes occurred in the electrocardiogram. The results of one experiment are shown in figure 1. The chief alterations are slowing of heart rate, prolongation of *P-R* interval, ectopic rhythm, secondary tachycardia, and finally, ventricular fibrillation. Similar data were obtained in the remaining 2 animals.

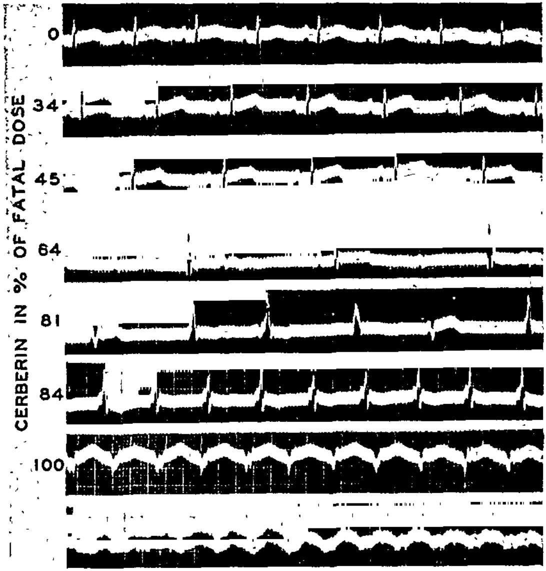

Fig. 1. Electrocardiographic Changes Produced by Cerberin

Cat, male, weighing 2.25 kgm., was anesthetized with ether. Cerberin in a 1:100,000 solution was injected into the left femoral vein at the rate of 1 cc. per minute. The cat died with a dose of 0.169 mgm. per kgm. A total of 33 electrocardiograms was taken from Lead II at different stages of the experiment. Only 8 selected tracings are shown in the figure. The abscissal time-lines make divisions of 0.02 and 0.1 second, and the ordinates represent 0.0001 and 0.0005 volt.

Vomiting uniformly took place when non-anesthetized pigeons and cats were

TABLE 1

*Assay in cats*

| COMPOUND | SEX | BODY WEIGHT | HEART WEIGHT | FATAL DOSE BY | | MEAN LETHAL DOSE ± S.E. BY | |
|---|---|---|---|---|---|---|---|
| | | | | Body weight | Heart weight | Body weight | Heart weight |
| | | *kgm.* | *grams* | *μgm. per kgm.* | *μgm. per gm.* | *μgm. per kgm.* | *μgm. per gm.* |
| Cerberin | F | 2.615 | 11.3 | 145.2 | 33.61 | 147.0 ± 5.7 | 40.44 ± 2.58 |
| | F | 2.618 | 7.0 | 130.6 | 48.86 | | |
| | M | 2.751 | 9.0 | 161.9 | 495.0 | | |
| | M | 2.332 | 8.8 | 150.5 | 39.89 | | |
| | M | 2.311 | 7.5 | 149.8 | 46.16 | | |
| | F | 2.118 | 7.4 | 132.9 | 38.04 | | |
| | M | 2.526 | 10.8 | 161.5 | 37.78 | | |
| | F | 2.641 | 9.6 | 176.4 | 48.54 | | |
| | F | 2.735 | 9.8 | 154.2 | 43.04 | | |
| | F | 2.768 | 12.3 | 116.0 | 26.10 | | |
| Cerberoside | F | 2.100 | 7.7 | 996.6 | 271.8 | 813.7 ±78 | 218.5 ±20.2 |
| | M | 2.688 | 10.0 | 729.2 | 196.0 | | |
| | M | 2.533 | 9.2 | 1550.7 | 427.0 | | |
| | F | 2.654 | 9.4 | 753.6 | 212.8 | | |
| | M | 2.396 | 8.7 | 583.5 | 160.7 | | |
| | F | 1.993 | 6.8 | 664.3 | 194.7 | | |
| | F | 2.481 | 9.8 | 790.8 | 200.2 | | |
| | F | 2.337 | 8.7 | 876.3 | 235.4 | | |
| | M | 3.013 | 11.0 | 553.6 | 151.6 | | |
| | M | 3.362 | 14.8 | 1007.7 | 228.9 | | |

TABLE 2

*Assay in frogs*

| GLYCOSIDE | DOSE | NUMBER IN SYSTOLE/NUMBER USED | $SD_{50}$ ± S.E. |
|---|---|---|---|
| | *μgm. per gm.* | | *μgm. per gm.* |
| Cerberin | 1.2 | 0/8 | 1.789 ±0.075 |
| | 1.6 | 2/10 | |
| | 2.0 | 8/10 | |
| Ouabain | | | 1.108 ±0.065 |
| Cerberoside | 5 | 0/5 | 16.57 ±1.19 |
| | 10 | 1/5 | |
| | 12 | 1/5 | |
| | 14 | 2/10 | |
| | 16 | 4/10 | |
| | 18 | 7/10 | |
| | 20 | 7/10 | |
| Ouabain | | | 0.465 ±0.011 |

given appropriate doses of cerberin or cerberoside. When applied to isolated rabbits' intestines and guinea pigs' uteri, both glycosides produced stimulation.

All the evidence on hand indicates conclusively that cerberin and cerberoside have a digitalis-like action.

In order to determine the potencies of the 2 compounds, assays were carried out in frogs by the U.S.P.XI method (14), in cats by the procedure customarily employed in this laboratory (15), and in pigeons by the Hanzlik technique (16). The following strengths of solutions were used: With cerberin, 1:100,000 for cats, 1:20,000 and 1:5,000 for frogs, and 1:10,000 for pigeons; with cerberoside, 1:25,000 for cats, 1:2,000 for frogs, and 1:1,000 for pigeons.

As shown in table 1, cerberin has a high potency, and is approximately 5 times as toxic as cerberoside to cats. The difference of activity between the 2 glycosides becomes much greater in frogs if ouabain is taken as the standard of comparison, as illustrated in table 2. In pigeons, however, cerberoside is only slightly less active than cerberin in producing vomiting (table 3). Such discrepancies of results in different species of animals are not uncommon in accordance with our previous experience (17).

TABLE 3

*Emesis in pigeons by intravenous injection*

| GLYCOSIDE | DOSE | NUMBER VOMITED/ NUMBER USED | EMD$_{50}$ ± S.E. |
|---|---|---|---|
| | *μgm. per kgm.* | | *μgm. per kgm.* |
| Cerberin | 50 | 0/5 | |
| | 100 | 0/5 | |
| | 125 | 3/5 | 126.7 ± 7.0 |
| | 150 | 4/5 | |
| | 170 | 3/3 | |
| Cerberoside | 100 | 1/10 | |
| | 125 | 5/10 | |
| | 160 | 3/10 | 150.2 ±11.6 |
| | 200 | 9/10 | |
| | 250 | 9/10 | |

## SUMMARY

1. Cerberin can be isolated from both the oil and the defatted kernels of *Cerbera odollam* nuts.
2. From a second batch of nuts, a glycoside similar to but not identical with cerberin has been isolated, to which the name of cerberoside has been proposed.
3. Both cerberin and cerberoside have a digitalis-like action. Cerberin is much more potent on the heart than cerberoside.

*Acknowledgment.* The authors are indebted to Messrs. Chester C. Hargreaves, William T. Winchester, E. Brown Robbins, Robert C. Anderson, Harold Worth, and Clarence E. Powell.

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# THE ACTION OF POSTERIOR PITUITARY EXTRACT UPON VARYING DEGREES OF WATER LOAD ADMINISTERED TO FROGS

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Theobald (1), Hart and Verney (2) and Pickford (3) demonstrated that the less the water load, the greater the relative water-retaining power of a given dose of posterior pituitary extract in mammals. From the data of Pickford (3), for example, it may be estimated that a dose of $5 \times 10^{-5}$ I.U. (International Units) of posterior pituitary extract per kilo body weight inhibited by about 30% the diuresis produced in dogs by the administration of water in amounts corresponding to 2% of their body weights, while the inhibition when only half this much water was given amounted to about 70%. In the present communication it will be shown that posterior pituitary extract in frogs retains progressively more water the greater the amount of water administered until a maximal retention is reached, after which the water-retaining power of the extract decreases rapidly.

The technique for varying water load was not identical to that of the authors noted above. They gave posterior pituitary extract at increasing intervals after giving the same amount of water by mouth and thus, they concluded, after progressively less water was left in the body. While it is true that under these circumstances the pituitary extract acted upon progressively smaller water loads, it is also the case that the distribution and effects of water taken by mouth may have varied during the varying intervals before the pituitary extract was given. In other words, water load cannot be considered strictly as the only factor which varied in their experiments. To overcome this objection in the present experiments, posterior pituitary extract and water were both given at the same time to frogs and the only variable was the amount of water load.

The technique followed was similar to that of Boyd and Whyte (4). Leopard frogs, *Rana pipiens*, were acclimatized to room temperature in water during the warm summer months. The required numbers of frogs were then each day individually removed from the tank, the urine expressed by pressure over the abdominal wall, the skin carefully dried and body weight determined to the nearest 0.1 gm. In 6 groups of 2 frogs each, they were given respectively 10, 8, 6, 4, 2 and 0 cc. of distilled water per 100 grams body weight by injection into the dorsal lymph sac. One frog of each pair in each group then received by subcutaneous injection 0.5 I. U. of Pituitrin, N.N.R., per 10 grams body weight and the other member of the pair was given an equivalent volume of pituitrin vehicle. Every frog received water with or without pituitrin at the same time, the time being noted, and was placed in a 400 cc. breaker covered with a weighted glass shield. The animals were then re-weighed at intervals of 0.5 hours from the time of administration of water and pituitrin or pituitrin vehicle. This experiment was repeated to a total of 84 frogs per group, half given pituitrin and half pituitrin vehicle.

Taking the loss of body weight as an index of the loss of body water, we then could calculate the mean loss of body water in terms of grams per 100 grams body weight of frog. By subtracting the loss of body water in frogs receiving no added water from the loss in frogs given added water, it was possible to calculate the loss of the injected water in terms of percent of the volume injected. These figures were calculated for those frogs which received and for those frogs which did not receive pituitrin and the difference indicated the amount of injected water which was being held in the body by pituitrin at each interval after injection. The amount of injected water which was thus being held or retained in the body by pituitrin was expressed as a percentage of the water injected and the mean figures for these values have been plotted in figure 1.

It may be seen from figure 1 that, as more water was injected, progressively more of the injected water was retained by pituitrin. Thus when water was injected at the rate of 2 cc. per 100 grams of frog, about ⅓ of it was retained in the body by pituitrin over a period of 4 to 5 hours. When 4 cc. were injected, about the same amount was retained but when 6 cc. were injected, an average of about 55% was retained and with 8 cc. the retention rose to almost 80% during the 4th hour. When greater amounts of water, 10 cc., were injected, the percentage retention began to fall off from the 8 cc. values.

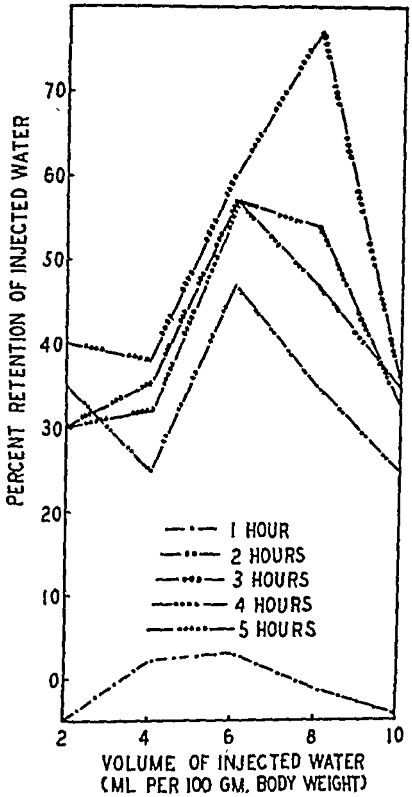

FIG. 1. THE PERCENTAGE RETENTION IN FROGS BY PITUITRIN OF VARIOUS AMOUNTS OF INJECTED WATER

From these data it was obvious that increasing amounts of injected water were retained by pituitrin to an increasing degree until a maximal retention was reached with some 6 to 8 cc. per 100 grams body weight. To discover the effect of pituitrin upon each additional amount of injected water, the percentage retention by pituitrin of each additional 2 cc. of injected water per 100 grams body weight was calculated at each hour and the means of these values have been assembled in table 1. From table 1 it is even more obvious that additional volumes of injected water are retained to a progressively greater degree up to the 3rd injection of 2 cc. The 5th injection of 2 cc. was not only not retained at all, but it hastened the loss of water which otherwise would have been retained by pituitrin. So to speak, the final additional water broke over the pituitrin dam and by virtue of the deluge carried away part of the dam which had been holding back the smaller volumes of water.

THE EFFECT OF VARYING THE DOSE OF PITUITRIN. The dose of pituitrin used in the above experiments corresponded to that used in similar studies on frogs, for example those of Boyd and Whyte (4). Per unit body weight, it was one million times the dosage used by Pickford (3) for example. Boyd and Whyte (4) investigated doses of pituitrin from $5 \times 10^{-1}$ to $2.5 \times 10^{-3}$ I.U. per 10 grams body weight of frog but all of these doses are much greater than those used in studying the antidiuretic action of pituitrin in mammals. It was not until studies of a wide range of doses of posterior pituitary extract upon water metabolism had been made in this laboratory by Boyd, Garand and Livesey (5), that the significance of such studies became apparent. Such studies of the effect of a wide range of doses of posterior pituitary extract upon water metabolism are lacking in most published reports. This is probably because posterior pituitary extract is available in ampules of 10 I.U. per cc. and doses of this order, when originally injected into man, gave rise to the antidiuretic reaction, while in frogs they produced the Brunn or water uptake reaction. Many of the effects of posterior pituitary extract upon body water are effects which would disappear or even be reversed with doses of the extract outside a relatively narrow range (5).

TABLE 1

*The percentage retention by pituitrin of each additional 2 cc. of water per 100 grams body weight injected into frogs*

| ADDITION | PERCENTAGE RETENTION (HOURS AFTER INJECTION) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | Mean |
| 1st | −5 | 35 | 30 | 40 | 30 | 26 |
| 2nd | 10 | 15 | 40 | 35 | 35 | 27 |
| 3rd | 5 | 90 | 100 | 105 | 105 | 81 |
| 4th | −15 | 0 | 20 | 130 | 45 | 36 |
| 5th | −15 | −15 | −130 | −130 | −50 | −45 |

To investigate the effects of a wide range of doses of pituitrin upon the reaction described above in frogs, the animals were assembled as before and given 5 cc. of water per 100 grams body weight. They were then injected with pituitrin in doses of from $10^{-11}$ to 1 I.U. per 10 grams body weight, each dose being contained in 0.1 cc. of pituitrin vehicle, which volume of vehicle was injected into controls receiving no pituitrin. Each dose was injected into a total of 20 frogs and the mean loss of body water again calculated as grams per 100 grams of frog and from these various figures the percentage retention by the various doses of pituitrin of the water injected.

The average percentage retentions have been collected in table 2. Doses below $10^{-2}$ I.U. per 10 grams body weight had no significant effect and hence they have been condensed as in table 2. It may be seen that the effect of pituitrin in delaying the loss of injected water in frogs was confined to relatively large doses of the drug. The maximal effect was obtained with a dose of 1 I.U. per 10 grams of frog, a dose which would correspond per unit body weight to the injection of a pint of posterior pituitary extract into an adult man. Indeed, it is possible that the reaction would have been more marked with even larger doses of pituitrin. For such doses pituitrin as ordinarily available cannot be used because its preservative, chlorbutol, begins to exert a lethal narcotic effect in doses of 5 I.U. per 10 grams body weight and over (6).

TABLE 2

*The percentage retention by a wide range of doses of pituitrin of 5 cc. of water per 100 grams body weight injected into frogs*

| DOSE OF PITUITRIN (I.U. PER 10 GMS.) | PERCENTAGE RETENTION (HOURS AFTER INJECTION) | | | | | |
|---|---|---|---|---|---|---|
| | 2nd | 3rd | 4th | 5th | 6th | Mean |
| 1 | 55 | 48 | 34 | 31 | 22 | 38 |
| $10^{-1}$ | 49 | 34 | 20 | 16 | 8 | 25 |
| $10^{-2}$ | 21 | 15 | 8 | 9 | 5 | 12 |
| $10^{-3}$ to $10^{-5}$ | −2 | −1 | −4 | −3 | −6 | −3 |
| $10^{-6}$ to $10^{-8}$ | 4 | 3 | −1 | 1 | −3 | 1 |
| $10^{-9}$ to $10^{-11}$ | 7 | 9 | 5 | 5 | 1 | 5 |

## SUMMARY

*Posterior pituitary extract, injected into frogs given increasing amounts of* administered water, retained progressively greater portions of each additional amount of water until a maximal effect was reached with a total injection of 6 to 8 cc. of water per 100 grams body weight. When further additions of water were given, the extract had little or no water-retaining effect on them.

A wide range of doses of posterior pituitary extract was then tried and it was found that the water-retaining effect of the extract was restricted to relatively large doses of the order of 1 international unit per 10 grams body weight.

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# THE PERCUTANEOUS ABSORPTION OF AMMONIUM HYDROGEN SULFIDE AND HYDROGEN SULFIDE[1, 2]

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It is well known that the local action of the more common alkaline sufides, when applied topically to the skin, results in a softening and subsequent destruction of the stratum corneum. Hydrogen sulfide itself, while having some local irritant action, may produce systemic poisoning due to its penetration of the skin. Although this fact is subject to some controversy, Walton and Witherspoon (1) have shown that the exposure of approximately one-half of the body surface of the guinea pig to pure hydrogen sufide gas proves fatal in about forty-five minutes. Basch (2) has cited cases in which poisoning resulted from the dusting of the skin or scalp with finely precipitated sulfur, and also of cases of poisoning in infants treated for scabies with a 10% precipitated sulfur ointment. This author believes that in conditions of an injured skin (either mechanically or from disease) sulfur is absorbed, which upon reduction produces hydrogen sulfide poisoning. On the other hand, Yant (3) has reported that the whole body of man may be exposed to 2% hydrogen sulfide for thirty minutes without observing symptoms of poisoning, discomfort, or skin discoloration. Schultze (4), in exposures of pure hydrogen sulfide gas to the intact skin of guinea pig, cat and man for periods up to sixty minutes, obtained skin discoloration but concluded that the absorption of the gas is too slow to cause serious systemic effects, and was unable to confirm the results of Walton and Witherspoon. In the case of ammonium hydrogen sulfide, little has been reported in the literature (Bunce, Parker, Lewis (5)) concerning either its local or systemic action.

EXPERIMENTAL. The present study on the local and systemic effects of ammonium hydrogen sulfide was undertaken because of the use by the trade of an aqueous solution of the compound in a so-called "cold-process" permanent hair wave. The material which was used in this study was a commercial hair wave preparation. It consisted of a clear, pale yellow, aqueous solution having a strong odor of hydrogen sulfide, a pH of 8.2, and an ammonium hydrogen sulfide content of 8.3%. In addition to the liquid preparation, hydrogen sulfide and ammonia gas alone and in combination were used. In these experiments the gases were conducted onto the skin which was kept moist by several layers of wet gauze. During the exposure the entire animal excepting the head was placed in a hood, and the draft of air was so regulated as to prevent any inhalation of the gases under test.

The mouse, guinea pig, rabbit, dog and man served as experimental subjects. The periods of exposure varied from a few minutes to approximately twenty hours. In the case

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[1] Read in part before The American Society for Pharmacology and Experiment Therapeutics, Boston, April 2, 1942.

[2] We wish to thank Dr. A. A. Nelson, Senior Pathologist, Food and Drug Administration, for the pathological examinations made in this study.

of the guinea pigs, rabbits and dogs the areas of skin exposures were prepared according to the following four categories, (*a*) clipped free of hair with the epidermis intact, (*b*) clipped with the epidermis abraded by careful incisions, (*c*) unclipped with epidermis intact, and (*d*) unclipped with epidermis abraded by incisions. The abrasions were longitudinal parallel incisions through the epidermis spaced 2 centimeters apart and extending over the entire area to be exposed. The purpose of these incisions was to simulate such breaks in the skin of the scalp as might result mechanically or from disease.

The exposure area was in the shape of a girdle extending around the middle of the trunk of the anmal; in the human, the exposure area of approximately 200 sq. cm. extended around the lower forearm. Contact of the test solution with the skin areas was maintained by means of a cuff made of thin dental rubber dam. This cuff was flared in the middle so as to provide a reservoir as well as free circulation of the fluid next to the skin. The edges of the cuff were constricted so as to form a snug elastic seal against the skin. During exposure all animals were immobilized. In the case of the rabbits a special holder was constructed which fastened the animal after the manner of a head and hip stock, but in such a way that it rested comfortable on its belly.

In addition to the skin applications the systemic effects of the administration of ammonium hydrogen sufide solution by other routes was studied. The material was applied orally, intravenously, intraperitoneally, intra- and subcutaneously to dogs, rabbits and mice.

During the various exposures lead acetate test paper was held in the stream of expired air of the subject, and the time noted at which initial darkening of the paper was obtained. In this manner it was possible to determine (qualitatively) the sulfide excretion by the lung, and also how soon after the beginning of the exposure sulfide is excreted by the lung.

The blood of rabbits exposed to ammonium hydrogen sulfide was examined for the presence of sulfhemoglobin. The method was briefly as follows: Blood from an ear vein was collected in a tube with oxalate and rotated while mixing to ensure complete oxygenation. A 0.5 cc. sample was transferred to a 25 cc. volumetric flask and hemolyzed with 10 cc. of water; 0.25 cc. of 1% sodium cyanide solution was added, and the volume made to 25 cc. The hemolysate was centrifuged forty minutes at 3000 R. P. M. to throw down stroma. Readings were made in a spectrophotometer at a wave length of 620 $M\mu$. The absorption cell was 10 cm. long and of all-glass construction. The method was sufficiently sensitive to detect sulfhemoglobin resulting from the reaction of 18 micrograms of ammonium hydrogen sulfide with one cubic centimeter of blood *in vitro*. This amount represents the concentration of ammonium hydrogen sulfide that would be present *in vivo* if 1.5 mgm. ammonium hydrogen sulfide were injected intravenously into the rabbit. (This if approximately the *LD 50*.) The calculation also assumes a blood volume of 80 cc./kg. for the rabbit. The amounts of sulfhemoglobin found were expressed in per cent saturation. Calibration curves relating per cent saturation with spectrophotometer readings were obtained from various mixtures of blood of zero and 100% saturation with respect to sulfhemoglobin.

Preparations of sulfhemoglobin were studied to determine their toxicity. Whole blood from rabbits were saturated with hydrogen sulfide in order to convert the hemoglobin into sulfhemoglobin. After removal of hydrogen sulfide remaining in physical solution, the blood was injected into a rabbit just previously bled of a comparable amount of normal blood. The dosage ranged from 1–13 cc. per kgm.

In an effort to obtain further information on the mechanism of the systemic action of ammonium hydrogen sulfide, methylene blue was administered to rabbits in the form of 0.5% solution in doses ranging from 2 to 8 mgm. per kilograms of body weight. Artificial respiration alone and in combination with methylene blue was also administered. In addition to these restorative measures animals which had been poisoned slowly by the topical application of ammonium hydrogen sulfide to the skin were given strychnine sulfate subcutaneously in conjunction with the methylene blue and artificial respiration.

Histological examinations were made of the important parenchymatous organs and of skin sections both from biopsy and post mortem specimens.

TABLE 1

*Topical skin application of an 8.3% aqueous solution of ammonium hydrogen sulfide*

| NO. OF SUBJECTS | RANGE OF BODY WT. | SKIN PREPARATION | LETHAL AMOUNT $NH_4HS$ SOLN. | DURATION OF EXPOSURE | REMARKS |
|---|---|---|---|---|---|
| | *kgm.* | | *cc.* | | |
| *Rabbit* | | | | | *Area exposed—240 (cm)² = 9.3% of body surface* |
| 5 | 2.3–3.6 | Clipped<br>Abraded | 3–5 | 3–12 min. | Death in all cases<br>Survival 24 hrs. on 5 cc. |
| 17 | 2.9–3.5 | Clipped<br>Intact | 5–20 | 8 hrs. | Average for 10 cc. was death in 8 hrs.<br>Death in 2¼ hrs. from 25 cc. |
| 3 | 2.6–2.8 | Unclipped<br>Abraded | 10–25 | 20 min. | Two survived 10 and 15 cc. |
| 14 | 2.8–3.2 | Unclipped<br>Intact | 10–60 | 20 min. to 20 hrs. | 20 cc. caused death in 8½–10 hrs.<br>60 cc. caused death in 26 min. |
| *Guinea pig* | | | | | *Area exposed—90(cm)² = 12.8% of body surface* |
| 5 | 0.7–0.8 | Clipped<br>Abraded | 1.5–5 | 20 min. to 2 hrs. for smallest dose | 3–5 cc. caused death 26–47 min.<br>g. pig survived from 1.5 cc. |
| 4 | 0.7–1.2 | Clipped<br>Intact | 2.5–10 | 20 hrs. | No deaths from 5 cc. or less. Above 5 cc. g. pigs died in 12–18 days from secondary causes due to exposures |
| 5 | 0.6–1.0 | Unclipped<br>Abraded | 5–20 | 20 min.–2 hrs. | Death in 2–3 hrs. from 10 cc. or more |
| 4 | 0.5–1.0 | Unclipped<br>Intact | 5–10 | 4–20 hrs. | Survival 24 hrs. 10 cc. but death from secondary causes up to 4 days. No deaths at 5 cc. |
| *Mouse* | | | | | *Area exposed—9 (cm)² = 8.2% of body surface* |
| 10 | 0.025–0.035 | Unclipped<br>Intact | 0.25–1.0 | 3–20 min. | More than 0.5 cc., death in 3–20 min.<br>Less than 0.5 cc., survival |
| *Dog* | | | | | *Area exposed—500 (cm)² = 4.6% of body surface* |
| 5 | 9–10 | Clipped<br>Intact<br>Unclipped<br>Intact<br>Unclipped<br>Abraded | 40–160 | 20 min.–3 hrs. | 116 cc. to clipped abraded skin produced minor symptoms of toxicity. No deaths |

RESULTS. A summary of the results of skin exposures in animals is given in table 1. It is apparent that ammonium hydrogen sulfide penetrated the intact skin of rabbits and mice and produced fatal results. The difference between

TABLE 2

*The penetration of hydrogen sulfide alone and in combination with ammonia through the rabbit skin*

| RABBIT NUMBER | SEX | WT. IN GRAMS | PREPN. OF SKIN* | GAS FOR EXPOSURE | DURATION OF EXPOSURE | REMARKS |
|---|---|---|---|---|---|---|
| 78 | ♂ | 3560 | Clipped<br>Intact | Hydrogen sulfide | 2 hrs. | Animal survived, but exhibited erythema and "slate grey" discoloration of skin exposed. Areas 1 cm. ± dark-chocolate colored. Expired air gave sulfide reaction with lead acetate test paper |
| 79 | ♂ | 3775 | Clipped<br>Intact | Ammonia plus hydrogen sulfide | $1\frac{1}{4}$ hrs. | Died 30 min. after termination of exposure from typical symptoms of $NH_4HS$ poisoning. Skin lesions. Intense erythema slate grey and usual areas 1 cm. + of chocolate-like discoloration |
| 80 | ♂ | 3060 | Clipped<br>Intact | Ammonia | 1 hr. 25 min. | Intense erythema. This exposure caused sufficient burns to skin to cause death 2 days after exposure |
| 81 | ♂ | 3405 | Clipped<br>Abraded | Hydrogen sulfide | 2 hrs. | Died in $2\frac{1}{4}$ hrs. Slate grey discoloration of skin and usual areas 1 cm. + in diameter of chocolate-colored areas |
| 82 | ♂ | 3200 | Clipped<br>Abraded | Ammonia plus hydrogen sulfide | 43 min. | Died in 49 minutes. Usual skin lesions. During exposure, lead acetate test paper gave strong sulfide test in expired air |
| 84 | ♂ | 3385 | Clipped<br>Intact | Hydrogen sulfide | $1\frac{1}{2}$ hrs. | Died in 1 hr. 40 min. Symptoms of toxicity noted 26 min. after initial of exposure. Sulfides in expired air 7 min. after initial of exposure |
| 85 | ♀ | 3790 | Clipped<br>Intact | Ammonia plus hydrogen sulfide | 48 min. | Died in 60 min. First symptom of toxicity 12 min. after initial of exposure. Sulfides in expired air 5 min. after initial of exposure |

* Areas of exposure measured approximately 240 (cm)$^2$.

abraded and intact skin is most striking. The presence of hair served to reduce the effective toxicity, possibly by adsorbing considerable quantities of the compound as well as preventing its intimate contact with skin, since death resulted only from a relatively large dose (25 cc.) when applied to the unclipped abraded skin of the rabbit. The same general relationships were also obtained with intact skin.

In table 2 are given the results of the application of hydrogen sulfide gas, alone and in combination with ammonia, to the skin of rabbits. It can be seen that the penetration of hydrogen sulfide alone is rather slow, but upon addition of ammonia the reaction is considerably enhanced. This difference is even more striking in the exposures of abraded skin to hydrogen sulfide. The exposure to ammonia gas alone caused severe burns of the skin. These burns were sufficiently severe, following one and one-half hours of exposure, to cause death within 24 hours.

TABLE 3

*Toxicity of ammonium hydrogen sulfide by various routes of administration*

| SUBJECT | ROUTE OF ADMINISTRATION | LETHAL DOSE $NH_4HS$ (MGM PER KGM. BODY WEIGHT) |
|---|---|---|
| Mouse | Intravenous | 2 |
| | Intraperitoneal | 10 |
| | Subcutaneous | 10 |
| | Oral | 80 |
| Rabbit | Intravenous | 1.5–2.0 |
| | Subcutaneous | 7.5–10 |
| | Intracutaneous | 30–40 |
| Dog | Intravenous | 2 |

In table 3 are given the lethal doses for the dog, rabbit and mouse when ammonium hydrogen sulfide is administered by routes other than percutaneous. Although not indicated in the table, it is interesting to note that intravenously the toxicity of hydrogen sulfide and ammonium hydrogen sulfide is the same when calculated on the basis of sulfur content. By comparison with Table 1, it can be seen that the intravenous dose is extremely small when compared with the percutaneous. In addition the intravenous lethal dose is approximately the same for all three species, while rather wide differences obtain in the percutaneous. This gives some indication of the measure of effectiveness of the epidermis and cutis in protecting the animal against toxic agents.

When the blood of rabbits was examined for sulfhemoglobin at various times during the exposure to ammonium hydrogen sulfide, the pigment could be clearly demonstrated only in those cases where the exposure had lasted for at least twenty minutes. In fact the highest percentages of saturation (15–20%) were observed after two or three hours, and on several occasions even after twenty-four hours. In contrast, where death resulted in 3–12 minutes after exposure of scarified skin, or even more quickly (2–3 minutes) after intravenous injection of ammonium hydrogen sulfide, no sulfhemoglobin was detectable in the blood.

In figure 1 the percentage saturation of blood has been plotted against time of exposure. It can be readily seen that the formation of sulfhemoglobin occurs rather slowly.

It seemed of interest to determine whether sulfhemoglobin, aside from its immobilization of oxygen-carrying pigments, could *per se* be toxic. When blood, completely saturated with hydrogen sulfide but with all traces of physically dissolved sulfide removed, was administered intravenously in the rabbit, no symptoms of toxicity were observed from doses of 1 to 13 cc. per kilogram of body weight. The only abnormality observed in these animals was the excretion of darkly colored urine for several days subsequent to the injection.

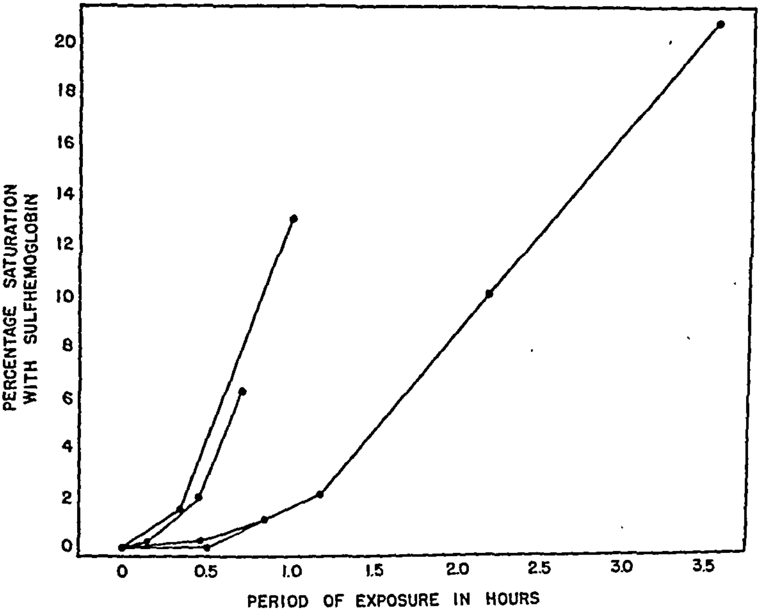

FIG. 1. THE INCREASE WITH TIME IN PERCENTAGE SATURATION OF RABBIT BLOOD WITH SULFHEMOGLOBIN DURING CUTANEOUS EXPOSURE TO AMMONIUM HYDROGEN SULFIDE

In table 4 are given the effects of the administration of methylene blue and artificial respiration, alone and in combination, in ammonium hydrogen sulfide poisoning. Methylene blue alone, in doses up to 8 mgm./kgm. intravenously, protects only one of five rabbits administered the surely fatal intravenous dose of 2 mgm./kgm of ammonium hydrogen sulfide. Artificial respiration alone protected rabbits from twice the surely lethal dose. Following combination of methylene blue and artificial respiration some animals were able to survive 2–3 times the surely fatal dose of 2 mgm./kgm.

Symptoms and pathology. In acute deaths the first symptom observed in ammonium hydrogen sulfide poisoning was a stimulation of respiration; the stimulation of the respiration was sometimes preceded by a slight drop in blood pressure. The amplitude as well as the rate of respiration was increased. As poisoning progressed there was dyspnea with a slowing of the rate to periods of apnea. As asphyxia became more pronounced the pupils were widely dilated

TABLE 4

*The effect of artificial respiration alone and with methylene blue in $NH_4HS$ poisoning*

| RABBIT NUMBER | SEX | WEIGHT | DOSE OF $NH_4HS$ IN MGM INTRAVENOUSLY/KGM.* | PROPHYLACTIC PROCEDURE† | REMARKS |
|---|---|---|---|---|---|
| | | *gms* | | | |
| 90 | ♀ | 4665 | 2 | 6 mgm./kgm. of methylene blue only | Recovered |
| 93 | ♂ | 3800 | 2 | 8 mgm./kgm. of methylene blue only | Recovered |
| 94 | ♀ | 4410 | 2 | 8 mgm./kgm. of methylene blue only | Died |
| 95 | ♂ | 3875 | 2 | 8 mgm./kgm. of methylene blue only | Died |
| 96 | ♂ | 3330 | 2 | Artificial respiration only | Recovered—no subsequent ill effects |
| 98 | ♂ | 3365 | 2.5 | Artificial respiration only | Recovered—no subsequent ill effects |
| 99 | ♂ | 4050 | 2.5 | Artificial respiration | Recovered but died 2 days later |
| 100 | ♂ | 4270 | 3 | Artificial respiration | Recovered |
| 102 | ♂ | 3615 | 4 | Artificial respiration only | Recovered—no subsequent ill effects |
| 103 | ♂ | 3340 | 4 | Artificial respiration plus 6 mgm./kgm. of methylene blue | Recovered—no subsequent ill effects |
| *104* | ♂ | *3960* | *5* | *Artificial respiration plus 6 mgm./kgm. of methylene blue* | *Recovered—no subsequent ill effects* |
| 105 | ♂ | 4040 | 6 | Artificial respiration plus 8 mgm./kgm of methylene blue | Recovered—no subsequent ill effects |
| 106 | ♂ | 3535 | 8 | Artificial respiration plus 6 mgm./kgm. methylene blue | Recovered—but sacrificed due to nerve injury |
| 108 | ♀ | 4150 | 8 | Artificial respiration plus 8 mgm./kgm. methylene blue | Recovered—but sacrificed due to nerve injury |
| 109 | ♀ | 2785 | 8 | Artificial respiration plus 8 mgm./kgm. methylene blue | Recovered—died 24 hours later due to nervous injury |

* The lethal dose by intravenous route for rabbit is 1.5–2.0 mg. $NH_4HS$/kgm.

† Methylene blue when administered was given intravenously.

and the animal appeared uneasy to the point of struggling or minor convulsions. The asphyxia was accompanied by a rise in blood pressure. The animal usually died, after complete prostration with or without a violent convulsion, from complete arrest of respiration. The heart continued to beat for two or three minutes following complete arrest of respiration.

In a few human subjects, where the forearm was exposed for ten to twenty minutes, the immediate effect was a local sensation of warmth, described by some as uncomfortable. Upon removal of the cuff there was also a purplish to "slate gray" discoloration of the skin, but this was evanescent. The most significant effect was an erythema which persisted for several days and was followed by pigmentation. The exposed area was only slightly painful to pressure and could be described as resembling a severe sunburn. It is rather interesting that a solution of ammonia applied in concentration equal to that in ammonium hydrogen sulfide but neutralized to approximately the same pH (8.2) with carbon dioxide was without noticeable subjective or objective effect upon the human skin.

After exposure to ammonium hydrogen sulfide or hydrogen sulfide the skin of animals also showed a purplish or slate gray discoloration. In many cases this was quite superficial and evanescent although accompanied by a dermatitis. Many small areas of one or more centimeters in diameter were of dark chocolate color and remained so. Histological examination of skin from six animals showed an acute necrotizing dermatitis of varying severity, up to complete necrosis of the epidermis, hair follicles and corium. The corium in general showed less damage than the epidermis. A moderate amount of acute inflammatory cellular exudate was present; edema was occasionally present. In addition, two animals which survived twenty-four and forty-six hours beyond the time of exposure to ammonium hydrogen sulfide showed numerous foci of recent coagulative necrosis of the liver and some kidney damage, consisting of slight necrosis of the loops and convoluted tubules and the presence of moderate amounts of protein material in the tubular lumens. In contrast, except for the usual superficial effects noted grossly on the skin, one animal which died within three minutes showed no pathological lesions attributable to the application of ammonium hydrogen sulfide.

Discussion. Ammonium hydrogen sulfide is a severe irritant capable of penetrating the intact skin and causing death. Evidence that the compound must reach the blood stream is clearly demonstrated by the findings of hydrogen sulfide in the expired air, sometimes considerably in advance of the onset of symptoms. Notwithstanding the assertion of Haggard (6) that sulfhemoglobin is not found *in vivo*, it has also been a common finding that the blood of animals whose skin was exposed to ammonium hydrogen sulfide carried from 10–20% saturation with sulfhemoglobin. It must be noted that this formation of sulfhemoglobin required at least twenty minutes of skin exposure to ammonium hydrogen sulfide before appreciable amounts could be detected in the blood. Presumably this delay was caused, not by the lack of hydrogen sulfide in the blood (for this occurred within three to six minutes after the beginning of exposure), but by some other mechanism such as methemoglobin formation. In connection with the slow formation of sulfhemoglobin, it is also interesting to note that when 2 mgm. per kgm. of ammonium hydrogen sulfide are injected intravenously and death results in from three to five minutes, no sulfhemoglobin can be detected, even though the addition of comparable amounts of ammonium hydrogen sulfide to blood *in vitro* produces enough sulfhemoglobin to be readily detectable.

The contrast in symptoms resulting from the application of small intravenous doses and large topical doses to the skin, is striking. In the former case death occurs almost instantaneously due to respiratory failure. The respiratory involvement closely resembles the effects of cyanide; in fact the observations of Heymans and Bouckaert (7) indicate that the hyperpnea of hydrogen sulfide, like that of cyanide is a chemoreceptor phenomenon. Methylene blue alone, and especially in combination with artificial respiration, enables the animal to withstand the respiratory crisis. It would appear that artificial respiration is merely the mechanical factor of maintaining the organism during the period of temporary paralysis of the respiratory center. In addition, strychnine was given also in combination with methylene blue and artificial respiration. Although strychnine is not considered a very satisfactory respiratory stimulant, it was given to these animals exhibiting sudden complete failure of the respiratory center to produce violent medullary stimulation thereby attempting to raise the reflex excitability of the center. It was hoped thereby to carry the animals beyond the acute crisis, which few survive. The function of methylene blue is less clearly defined. With the slow death, following topical skin application, the animal exhibits unmistakable signs of oxygen lack. This may be caused (*1*) by a deterioration of the mechanism of oxygen utilization in the tissues or (*2*) disturbances in the oxygen carrying capacity of hemoglobin. It would appear that a 20% saturation of blood with sulfhemoglobin could scarcely be the sole factor involved here, and certainly the indications are that sulfhemoglobin itself is non-toxic.

The percutaneous absorption of hydrogen sulfide alone is relatively slow; the presence of abrasions on the skin, however, permits relatively rapid absorption. In the case of ammonium hydrogen sulfide, absorption is relatively rapid even through the intact skin. These experiments do not show why ammonium hydrogen sulfide penetrates the skin more rapidly. The irritating properties of the ammonium radical may in part account for the erythema and congestion of the skin. This could favor absorption because of the increased blood supply to the part. On the other hand, the intrinsic properties of molecular ammonium hydrogen sulfide may be such as to favor its penetration over that of molecular hydrogen sulfide.

## CONCLUSIONS

(1) Ammonium hydrogen sulfide is a severe skin irritant; it penetrates the intact skin producing characteristic symptoms and death.

(2) Abrasions of the skin facilitate the absorption of ammonium hydrogen sulfide.

(3) Poisoning from ammonium hydrogen sulfide occurs more rapidly than from hydrogen sulfide, when these compounds are in contact with the skin.

(4) Following skin exposures to either ammonium hydrogen sulfide or hydrogen sulfide, sulfhemoglobin can be detected in the blood and hydrogen sulfide in the expired air.

(5) Artificial respiration, alone and in combination with methylene blue, is effective in protecting animals against two to three times the intravenous lethal dose of ammonium hydrogen sulfide.

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## FEDERATION PROCEEDINGS

The Federation of American Societies for Experimental Biology, composed of The American Physiological Society, The American Society of Biological Chemists, The American Society for Pharmacology and Experimental Therapeutics, The American Society for Experimental Pathology, The American Institute of Nutrition and The American Association of Immunologists, has begun (1942) the publication of the Federation Proceedings.

Four issues will be published annually. Each year the *March* issue will contain the complete Federation Program of the scientific sessions of all the component Societies as prepared for the forthcoming annual meeting with abstracts of all scientific papers to be presented; the *June* and *September* issues will contain the full text of twenty or more papers presented at the annual meeting, including probably the papers on the joint society program and papers of several society symposia; the *December* issue will contain material pertinent to the Federation membership, i.e., the officers, membership list, together with an index of the completed volume.

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# THE PARENTERAL USE OF ORGANIC ESTERS

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Vegetable oils have been employed as solvents for the water-insoluble, antihemorrhagic *2*-methyl-*1*,*4*-naphthoquinone (Menadione, N. N. R.) but, although parenteral administration has been successful, experience has shown that the natural oils are not entirely satisfactory for such use. In sunlight, or on irradiation with strong artificial light, the commonly employed oils, (peanut, corn and almond) deteriorated rapidly at 100°C., and the absorption curves[1] were altered markedly, even at room temperature. Like aqueous solutions of the quinone, those in oils were also photosensitive. Furthermore, these oils are absorbed rather slowly when administered intramuscularly. Considerable amounts of corn oil remained at the site of injection in guinea-pigs for three days and the same was true of almond oil, though it seemed to be absorbed somewhat more rapidly. Accordingly, a search was made for solvents that were not only stable towards light but were also more readily absorbed.

Since liquid esters of organic acids proved to be suitable solvents for the quinone, 25 such esters were injected into the gluteal muscles of guinea-pigs and the animals were carefully examined for systemic toxicity, local irritation, and the action upon the neuro-muscular functions of the injected leg.

Experimental. Ether solutions of the commercially available esters were washed successively with cold saturated sodium bicarbonate solution and water, the solvent was distilled from the dried, filtered ether solution and the residue was fractionally distilled *in vacuo*. The remaining esters were prepared by appropriate methods, either from the acid chloride and the required alcohol, or by esterification of the acid with 5–10% of sulphuric acid as the catalyst. The resulting esters were purified as above described.

Ethyl-*N*-diethylglycine was prepared from ethyl chloroacetate and diethylamine in the usual manner and distilled.

The monobutyl ester of succinic acid was isolated from a large-scale preparation of the normal ester by extraction of the crude product with cold saturated sodium bicarbonate solution, an ether solution of the ester liberated on acidification was dried, the solvent was distilled from the filtered solution and the residual ester was fractionally distilled in vacuo. The properties of the esters are summarized in table 1.

Monosodium succinate was prepared by adding an alcoholic solution of the acid to one equivalent of sodium ethylate in ethanol and the precipitated salt (anal. calcd. for $C_4H_5O_4$ Na; Na, 16.41, found: 16.80) was dried in vacuo. A solution of 3 grams of the salt in 10 cc. of water was sterilized and used in the toxicity tests.

For the toxicological experiments 0.5 cc. or 1.0 cc. of the sterilized esters was injected with a short fine needle into the upper part of the leg of the test animal. The animals were observed continuously for several hours following the injection and were then examined daily for a week. In these examinations, the respiration, free-walking and locomotor

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[1] The spectroscopic measurements were made by Dr. D. Richardson of the Stamford Laboratories.

TABLE 1

| NO. | SUBSTANCE | B.P. AT MM. | | INDEX OF REFRACTION | DOSE | SYSTEMIC SYMPTOMS | SYMPTOMS IN INJECTED LEG | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Deterioration of functions | Muscle toughness |
| | | | | $n_D^{20}$ | *cc./kg.* | | | |
| 1 | Glycerin triacetate (triacetin) | 143 | 12 | 1.4309 | 1.5 | Dyspnea, death | | |
| 2 | *n*-Butyl acetate | 125 | 760 | 1.3943 | 3.0 | Animals very weak, 25% died | +++ | +++ |
| 3 | Ethyl lactate | 152 | 760 | 1.4130 | 1.0 | Respiration labored, dyspneic | + | Edematous, discolored |
| | | | | | 2.5 | Dyspnea, death | | |
| 4 | Isopropyl lactate | 54 | 13 | 1.4099 | 1.25 | General lack of muscular control | ++++ | |
| | | | | | 2.5 | Animals very weak, 50% died | ++++ | |
| 5 | Ethyl α-hydroxy-isobutyrate | 146 | 760 | 1.4088 | 1.2 | Dyspnea, animals spasmodic | ++++ | |
| | | | | | 2.2 | Animals died | | |
| 6 | *n*-Butyl citrate | 176 | 2 | 1.4450 | 3.0 | | + | ++ |
| 7 | *n*-Butyl fumarate | 106 | 1 | 1.4461 | 3.0 | All animals died | | +++ |
| 8 | *n*-Butyl crotonate | 70 | 12–15 | 1.4320 | 3.2 | 75% of the animals died | ++++ | ++++ |
| 9 | Ethyl succinate | 95 | 10 | 1.4196 | 1.5 | | + | ++ |
| 10 | *n*-Propyl succinate | 82 | 2 | 1.4255 | 1.1 | | + | − |
| 11 | Isopropyl succinate | 66–67 | 2 | 1.4182 | 1.1 | | + | − |
| 12 | *n*-Butyl succinate | 103 | 2 | 1.4308 | 1.1 | | − | − |
| | | | | | 2.2 | | (+) | − |
| | | | | | 3.0 | | (+) | +, some edema |
| 13 | *n*-Amyl succinate | 142 | 1 | 1.4340 | 3.2 | | − | ++ |
| 14 | Pentyl-*2*-succinate | 105 | 1 | 1.4287 | 3.0 | | + | ++ |
| 15 | Pentyl-*3*-succinate | 115 | 1 | 1.4360 | 3.0 | | (+) | ++ |
| 16 | *n*-Hexyl succinate | 165 | 1 | 1.4390 | 3.0 | | (+) | +++ |
| 17 | Cyclohexyl succinate | 163 | 1 | 1.4742 | 2.8 | | − | ++ |
| 18 | Ethyl pimelate | 98 | 2 | 1.4300 | 1.25 | | | |
| | | | | | 2.5 | | + | |

TABLE 1—*Continued*

| NO. | SUBSTANCE | B P AT MM | | $n_D^{20}$ | DOSE | SYSTEMIC SYMPTOMS | SYMPTOMS IN INJECTED LEG | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Deterioration of functions | Muscle toughness |
| | | | | | *cc./kg* | | | |
| 19 | Ethyl benzoate | 92 | 12 | | 1.25 | | ++ | ++ |
| 20 | $n$-Butyl benzoate | 89 | 2 | 1.4962 | 3.0 | | + | ++ |
| 21 | Cyclohexyl benzoate | 110 | 1 | 1.5220 | 3.0 | | ++ | ++ |
| 22 | Ethyl phthalate | 112 | 1 | 1.5015 | 1.25 | | ++ | ++ |
| 23 | $n$-Butyl phthalate | | | 1.4929 | 1.25 | | ++ | ++, loss in elasticity |
| 24 | $\gamma$-Valerolactone | 54–56 | 2 | 1.4340 | 2.5 | 75% of the animals died within 2 days | ++++ | ++++ |
| | 30% mono-sodium succinate | | | | 3.0 | 25% of the animals died | ++++ | +, edematous |
| 25 | Mono-butyl succinate | 139 | 2 | 1.4379 | 3.0 | Dyspnea, blood in urine, most animals died | ++++ | ++, later necrotic |
| 26 | Ethyl $N$-diethyl glycine | 82 | 25 | 1.4023 | 0.5 | | ++ | ++, edematous |

| | *Calculated for* | | | *Found* | |
|---|---|---|---|---|---|
| 7 | $C_{12}H_{20}O_4$ | C 63.15 | H 8.77 | C 63.1 | H 8.82 |
| 8 | $C_8H_{14}O_2$ | 67.6 | 9.9 | 67.54 | 9.9 |
| 14 | $C_{14}H_{24}O_4$ | 65.2 | 10.4 | 65.1 | 9.97 |
| 15 | $C_{14}H_{24}O_4$ | 65.2 | 10.4 | 65.12 | 10.42 |
| 16 | $C_{16}H_{30}O_4$ | 67.1 | 10.5 | 66.92 | 10.32 |

Only the values for esters not reported in the literature are recorded, with the compounds numbered as above.

reflexes were observed as well as the readiness and force with which the stretched leg was retracted. Changes in body weight and anatomical conditions at the site of injection were recorded. The results are summarized in table 1.

From table 1 it appears that the esters of the aliphatic, monocarboxylic acids with relatively low molecular weight are systemically toxic. The lethal dose of 1.5 cc./kg. of glycerine triacetate (triacetin) found in these experiments for guinea-pigs is not greatly different from that recently reported ($LD_{50}$ 2.3) (1) for white mice after subcutaneous administration. The behavior of the animals suggested that the lethal effect might be attributable to the large amounts of the acids liberated by the relatively rapid hydrolysis of these esters. The high toxicity of the acid monosodium salt as well as the monobutyl ester of succinic acid also confirms this view, but in the absence of exact knowledge of the fate of the neutral esters upon absorption the evidence is not definite.

The butyl esters of both the unsaturated acids examined, fumaric and crotonic, proved highly toxic and inapplicable for intramuscular use.

The closely related succinates showed slight differences in pharmacological action, though no outstanding differences were apparent between esters derived from normal and branched chain alcohols, nor between the esters of alcohols of even and uneven numbers of carbon atoms. The neutral succinates, as far as examined, did not show systemic toxicity, but whereas the ethyl and propyl succinates appeared to affect neuromuscular functions somewhat more strongly than the higher esters, the amyl and hexyl esters definitely produced the greatest damage to the musculature. The neutral butyl succinate showed the minimum toxicity and was applicable for parenteral use. Ethyl pimelate was equally satisfactory. The esters of the hydroxyacids, however, proved systemically toxic as did $\gamma$-valerolactone, the single lactone examined. The lethal dose 2.5

TABLE 2

| PATIENT | | DIAGNOSIS | TREATMENT WITH 2-METHYL-1,4-NAPHTHOQUINONE DAILY-INJECTED[3] | PROTHROMBIN TIME | |
|---|---|---|---|---|---|
| No. | Age | | | Hours after first treatment | In seconds |
| | | Ethylsuccinate (2 mg. k./cc.) | | | |
| 1 | 63 | Acute perforation of gangrenous gall bladder. Cholelithiasis | 1 | 0 | 19 |
| | | | 1 | 15½ | 16 |
| | | | | 39½ | 14 |
| 2 | 50 | Carcinoma of gall bladder with metastasis in liver | 1 | 0 | 39 |
| | | | | 29 | 21 |
| | | | 1 | 49 | 16 |
| | | | 1 | 74 | 15 |
| | | Butyl succinate (4 mg. k./cc.) | | | |
| 3 | 60 | Periarteritis nodosa | 0.5 | 0 | 27 |
| | | | | 14½ | 14 |
| 4 | 34 | Pulmonary tuberculosis | 0.5 | 0 | 37 |
| | | | 0.5 | 24 | 21 |
| | | | | 36 | 17 |
| 5 | | Obstructive jaundice. Secondary Ca. of liver | 0.5 | 0 | 35 |
| | | | | 18¼ | 14 |
| 6 | 42 | Obstruction of common bile duct by malignant glands | 0.5 | 0 | 37 |
| | | | | 1 | 29 |
| | | | | 3½ | 20 |
| | | | | 18¼ | 16 |
| 7 | | Obstruction of common bile duct by malignant glands | 0.5 | 0 | 35 |
| | | | 0.5 | 6 | 18 |
| | | | 0.5 | 22 | 13 |

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[3] Similar results were obtained by the oral administration of Vitamin K.

cc./kg. of ethyl lactate found by us on the guinea-pig is similar to $LD_{50}$ found by Latven and Molitor (2) for subcutaneous administration on the white mouse. Ethyl *N*-diethylglycine injured the musculature at the site of injection and esters of the amino acids were therefore not further examined. Similarly both *n*-butyl and cyclohexyl benzoates were injurious; the same was true for ethyl and butyl phthalate, and none of the examined aromatic esters proved useful. These results show that the applicability of esters for parenteral administration is limited to relatively few compounds and that the specificity is marked.

In clinical tests,[3] ethyl, butyl and cyclohexyl succinates were found to be harmless even when injections up to 2 cc. were made. No untoward effects were observed with solutions of the quinone in the esters. The anti-hemorrhagic activity of the solutions is demonstrated in table 2.

## SUMMARY

1. The applicability of organic esters as solvents for water-insoluble preparations administered parenterally has been tested.

2. Twenty-five liquid organic esters have been systematically examined for systemic toxicity, local irritation and the action upon neuromuscular functions when injected into the gluteal muscles of guinea-pigs.

3. Butyl succinate and ethyl pimelate proved least toxic and were suitable for parenteral use.

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[3] The clinical investigations were carried out by Dr. R. F. Farquharson, Toronto General Hospital; Dr. J. G. Allen, Albert Billings Memorial Hospital, University of Chicago, and Dr. Elmer Alpert, New York University College of Medicine, and the Medical Service of the Psychiatric Division of Bellevue Hospital, all of whom furnished us with reports of their findings. Table 2 is an abbreviated form of the data presented by Dr. Farquharson.

# THE ANTICONVULSANT ACTION OF DIPHENYL HYDANTOIN AND SOME RELATED COMPOUNDS[1]

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The first description of the usefulness of "Luminal" in the therapy of epilepsy by Hauptmann (1) carried no suggestion of the specific value of this derivative of barbituric acid, in comparison with others. When this specificity was realized,[2] the indication for trial of diphenyl barbituric acid was apparent, but attempts to prepare this substance were defeated by the failure of diphenyl malonic ester to condense with urea (3). When McElvain (4) finally prepared diphenyl barbituric acid from alloxan and benzene, this substance attracted little interest, since it was described as having no hypnotic properties.

The subsequent studies of Putnam and collaborators (5, 6, 7), which led to the introduction of diphenyl hydantion into the therapy of epilepsy, demonstrated the fundamentally important fact that a substance need not produce widespread depression of the central nervous system, such as results in sleep or anesthesia, to alleviate the convulsive disorder, epilepsy. Putnam's investigations also confirm the particular attributes of phenyl-substituted compounds.

DETERMINATION OF ANTICONVUSANT ACTION. Our experiments have been with convulsions produced electrically in cats with an apparatus like that of Putnam (5). The commutator gave a frequency of 105 per second, with an "on" period of 6.5 milliseconds. The convulsive threshold was taken as that current which, when applied for 10 seconds, led to the development of convulsive manifestations, especially leg movements, which continued after the cessation

*Cat no. 6*

| | | |
|---|---|---|
| 11:25 | 25 milliamperes. | No convulsion after stimulation. |
| 11:30 | 30 milliamperes. | No convulsion after stimulation. |
| 11:36 | 35 milliamperes. | No convulsion after stimulation. |
| 11:41 | 40 milliamperes. | Violent convulsions for 50 seconds after stimulation. |
| 12:40 | 35 milliamperes. | No convulsion after stimulation. |
| 12:45 | 40 milliamperes. | Brief convulsion after stimulation. |
| 1:48 | 40 milliamperes. | Convulsion for 47 seconds after stimulation. |
| 3:05 | 35 milliamperes. | No convulsion after stimulation. |
| 3:10 | 40 milliamperes. | Brief extensor spasm after stimulation. |
| 4:16 | 40 milliamperes. | Violent convulsion for 76 seconds. |
| 5:16 | 40 milliamperes. | Violent convulsion for 90 seconds. |
| 6:16 | 40 milliamperes. | Brief extensor spasm after stimulation. |

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[1] This investigation has been aided by a grant from G. D. Searle and Company, Chicago.

[2] Documentation of this point is lacking, although the statement can be found in textbooks. Dr. W. G. Lennox found less favorable results with Amytal than with phenobarbital (2). Others have doubtless had similar experience.

of the current. This threshold remained quite constant when redetermined hourly, as seen in the following protocol. Since five minutes were allowed between each period of stimulation, and since the threshold was somewhat elevated for a time after a convulsion, the value of the threshold stimulus was often determined only within ten milliamperes.

We have also studied the related compounds *5,5* diphenyl *2*-thiohydantoin, diphenylacetyl urea, and *5,5* diphenyl barbituric acid. Previous observations (8) suggested the study of *5,5* diphenylene hydantoin,[3] and fluorenone, Putnam *et al.* having demonstrated the anticonvulsant action of benzophenone. These compounds were all prepared by R. R. Burtner at G. D. Searle & Co. in Chicago. Table 1 presents the comparative date obtained with these compounds in 8 cats. The diphenyl hydantoins and barbituric acid, and the diphenylene hydantoin were injected intraperitoneally, as solutions of their sodium salts. The ureide and fluorenone were given by stomach tube.

The diphenyl-substituted acetylurea and thiohydantoin seemed devoid of anticonvulsant activity. The diphenyl barbituric acid and diphenylene hydantoin are about equally active and less so than diphenyl hydantoin. In table 2 is shown the acute toxicity of these compounds following intraperitoneal injection in rats. Diphenyl barbituric acid is about as toxic as diphenyl hydantoin, diphenylene hydantoin less toxic. The effects of the daily administration of diphenyl and diphenylene hydantoins were compared by giving 100 mgm. per kgm. of the former and 200 mgm. per kgm. of the latter to 10 rats each for 30 days. The weights of the rats, observed every fifth day, are shown in table 3, in comparison with controls. At the end of the period all rats were killed and their hearts, livers, kidneys, spleens, stomachs, and small intestines were examined histologically. No pathological changes attributable to the drugs were found. While the growth of the rats receiving diphenyl hydantoin was normal, that of those receiving diphenylene hydantoin was retarded. Since this substance is less than half as effective as an anticonvulsant than the diphenyl compound, but more toxic in twice the dose, it seems not to hold promise of clinical usefulness.

THE NATURE OF THE ANTICONVULSANT ACTION. As Putnam and others have reported, the response to diphenyl hydantoin (also diphenyl barbituric acid and diphenylene hydantoin) is not sleep or anesthesia. Large doses produce, rather, convulsive phenomena in rats, rabbits, and dogs. One dog receiving 0.2 gram per kgm. intraperitoneally showed loss of posture, increased reflex excitability, and slight convulsions. Twenty-four hours later there was extensor rigidity resembling that seen in the decerebrate animal. The knee jerks were greatly augmented. The next day, erect posture was maintained but rigidity was present and knee jerks were hyperactive. Consciousness was apparently present throughout the course. Similar results were seen in rabbits receiving up to 1 gram per kgm.

The response of the flexion reflex to these compounds was studied in two dogs

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[3] This compound was recently described by Henze and Speer, J. Am. Chem. Soc., 64: 523, 1942.

*Convulsive threshold stimuli in milliamperes*

| CAT NO. | DOSE | DIPHENYL HYDANTOIN | | | | | DIPHENYL BARBITURIC ACID | | | | | DIPHENYLENE HYDANTOIN | | | | DIPHENYL THIO HYDANTOIN | | | | DIPHENYL ACTYL UREA | | | FLUORENONE | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hours | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 0 | 2 | 4 | 6 | 8 | 0 | 2 | 4 | 6 | 24 | 0 | 2 | 4 | 6 | 0 | 2 | 4 | 6 | 0 | 2 | 4 | 0 | 2 | 4 | 6 | 24 |
| | mgm. per kgm. | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 20 | 40 | 100 | | | | | | | | | | | | | 40 | 50 | | | | | | | | | | |
| | 50 | | | | | | 50 | 90 | 80 | 80 | | | | | | | | | | | | | | | | | |
| | 80 | | | | | | | | | | | 35 | 70 | 60 | 60 | | | | | | | | | | | | |
| | 100 | | | | | | | | | | | 35 | 100 | 100 | 60‡ | | | | | | | | | | | | |
| | 200 | | | | | | | | | | | | | | | | | | | | | | 50 | 60 | 50 | 60 | |
| 2 | 20 | 30 | 40 | 40 | 40 | | 40 | 50 | 50 | 50 | | | | | | | | | | | | | | | | | |
| | 40 | | | | | | | | | | | 30 | 40 | 40 | 40 | | | | | | | | | | | | |
| | 50 | | | | | | 30 | 50 | 40 | 40 | | | | | | | | | | | | | | | | | |
| | 60 | | | | | | | | | | | | | | | 30 | 30 | 20 | 20 | | | | | | | | |
| | 200 | | | | | | | | | | | | | | | | | | | | | | 30 | 30 | 40 | 30 | |
| | 500 | | | | | | | | | | | | | | | | | | | | | | 30 | 30 | 70 | 80 | |
| 3 | 20 | 60 | 100 | 100 | 100 | | 40 | 70 | 80 | 60 | | | | | | | | | | | | | | | | | |
| | 40 | | | | | | | | | | | 50 | 80 | 90 | 90 | 40 | 50 | 50 | 50 | | | | | | | | |
| | 50 | | | | | | 50 | 100 | 90 | 90 | | | | | | | | | | | | | | | | | |
| | 200 | | | | | | | | | | | | | | | | | | | | | | 55 | 60 | 60 | 60 | |
| | 500 | | | | | | | | | | | | | | | | | | | | | | 40 | 50 | 50 | 100 | 70 |
| 4 | 20 | 50 | 100 | 100 | 100 | | 50 | 70 | 80 | 70 | | | | | | 50 | 50 | 50 | 50 | | | | | | | | |
| | 40 | | | | | | | | | | | 60 | 80 | 100 | 90 | | | | | | | | | | | | |
| | 50 | | | | | | 50 | 100 | | 100 | 70 | | | | | | | | | | | | | | | | |
| | 200 | | | | | | | | | | | | | | | | | | | | | | 60 | 80 | 80 | 70 | |
| | 500 | | | | | | | | | | | | | | | | | | | | | | 50 | 70 | 70 | 100 | 100 |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 20 | 50 | 100 | 100 | 90 | | | | | | | | | | | 50 | 50 | 50 | 50 | | | | | | | | |
| | 40 | | | | | | | | | | | 50 | 90 | 70 | 70 | | | | | | | | | | | | |
| 6 | 5 | 40 | 60 | 55 | 50 | | | | | | | | | | | | | | | | | | | | | | |
| | 10 | 30 | 60 | 50 | 60 | 60 | | | | | | | | | | | | | | | | | | | | | |
| | 20 | 35 | 90* | | | | | | | | | | | | | | | | | | | | | | | | |
| | 40 | | | | | | | | | | | | | | | 30 | 40 | | | | | | | | | | |
| | 60 | | | | | | | | | | | 35 | 55 | | | | | | | | | | | | | | |
| 7 | 10 | 40 | 60 | 55 | 45 | | | | | | | | | | | | | | | | | | | | | | |
| | 20 | 35 | 70 | 60 | 45 | 35 | | | | | | | | | | | | | | | | | | | | | |
| | 30 | | | | | | | | | | | 30 | 40* | | | | | | | | | | | | | | |
| | 1000 | | | | | | | | | | | | | | | | | | | 45 | 45 | 45 | | | | | |
| 8 | 20 | 40 | 66 | | | | | | | | | | | | | | | | | | | | | | | | |
| | 60 | | | | | | | | | | | | | | | 30 | 40 | 30 | | | | | | | | | |
| | 200 | | | | | | | | | | | 35 | | 100† | 40‡ | | | | | | | | | | | | |
| | 500 | | | | | | | | | | | | | | | | | | | 45 | 45 | | | | | | |

* 3 hours.
† 5 hours.
‡ 45 hours.

made spinal by transection of the cord at the first cervical vertebra, one decerebrate dog, and four dogs anesthetized with barbital, before and after spinal transection at the twelfth thoracic vertebra. Contraction of *M. tibialis anticus* was elicited by application of single shocks or brief periods of 60 cycle A.C. to *N. tibialis posterior*; the tendon of the muscle was attached to a torsion wire isometric lever, which wrote on a smoked drum. The flexion reflex was not influenced by doses adequate to raise the convulsive threshold (fig. 1). Two experiments with perfusion of the spinal subarachnoid space by the technique of Merlis and Lawson (9) gave inconclusive results.

TABLE 2

*Acute toxicity in rats*

| DOSE | DIPHENYL HYDANTOIN | DIPHENYL BARBITURIC ACID | DIPHENYLENE HYDANTOIN | DIPHENYL THIOHYDANTOIN | DIPHENYL* ACETYL UREA | FLUORENONE* |
|---|---|---|---|---|---|---|
| *gm. per kgm.* | | | | | | |
| 0.10 | | | | 0/4 | | |
| 0.25 | 0/4 | 0/5 | | 2/8 | | |
| 0.40 | | | | 6/6 | | |
| 0.50 | 3/6 | 2/6 | 0/10 | 6/6 | | |
| 0.75 | 6/6 | 6/6 | 1/6 | | | |
| 1.00 | 4/4 | | 9/11 | | | |
| 1.25 | | | 6/6 | | | |
| 1.50 | | | 4/4 | | | |
| 4.00 | | | | | 0/4 | 0/4 |
| 6.00 | | | | | | 0/4 |

* These two compounds given by stomach tube; the others intraperitoneally. Mortality ratios = dead animals/animals used.

TABLE 3

*Chronic toxicity in rats*

| DAY | AVERAGE WEIGHT IN GRAMS OF TEN RATS EACH | | |
|---|---|---|---|
| | Control | Diphenyl hydantoin | Diphenylene hydantoin |
| 0 | 95 | 97 | 95 |
| 5 | 115 | 118 | 106 |
| 10 | 132 | 133 | 117 |
| 15 | 148 | 151 | 125 |
| 20 | 160 | 160 | 137 |
| 25 | 172 | 173 | 150 |
| 30 | 179 | 187 | 163 |

The anticonvulsant action of diphenyl hydantoin was studied in four cats decerebrated at the level of the anterior corpora quadrigemina by the Sherrington guillotine. The convulsive phenomena were considerably modified by decerebration, in that thresholds were higher, and the movements consisted of extreme extension of the limbs, with coarse tremors, followed by running movements, rather than the synchronous clonus seen in the intact animals. These phenomena were not modified by the injection of diphenyl hydantoin in doses which had exhibited anticonvulsant action in these animals before decerebration.

The influence of diphenyl hydantoin on convulsions caused by cocaine and strychnine was studied in rabbits. Four rabbits received 80 mgm. per kgm. of cocaine hydrochloride, four 0.4 mgm. per kgm. of strychnine sulfate, all subcutaneously. All animals had typical convulsive attacks. Seven days later, these injections were repeated two hours after the intraperitoneal injection of 50 mgm. per kgm. of diphenyl hydantoin sodium salt. No anticonvulsant action

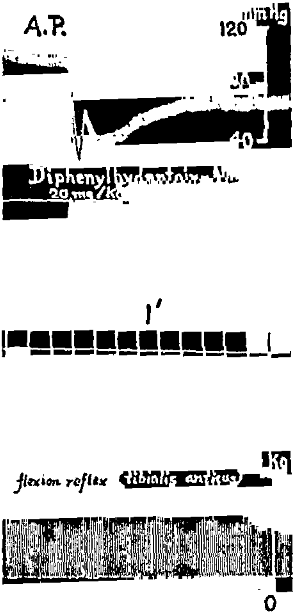

FIG. 1. Dog, barbital anesthesia. From the top down, carotid arterial pressure, signal of injection, time in minutes, flexion reflex of *M. tibialis anticus*. 60 cycle A.C. applied to *N. tibialis posterior* for 0.233 sec. every six seconds. At signal, injection into femoral vein of 20 mgm. per kgm. of diphenyl hydantoin sodium salt.

was apparent, in fact, two rabbits receiving cocaine and one receiving strychnine succumbed.

A similar experiment was performed, in which a course of seven days of twice-daily doses of 30 mgm. per kgm. of diphenyl hydantoin replaced the single dose. The convulsive phenomena were not altered.

EXCRETION OF DIPHENYL HYDANTOIN. Recovery of this substance added to urine is readily accomplished by acidification and extraction with ether. From the urine of three rabbits given 1 gram per kgm. orally, some extractive was occasionally found over a seven day period, but in amounts too small to permit identification. None could be found in the urine of three dogs receiving 0.1–0.2 gram per kgm. intraperitoneally, on examination for seven days.

DISCUSSION. The earliest symptomatic relief of epilepsy was accomplished with the bromides, substances that reduce the frequency and severity of convulsions but have a sedative rather than a soporific action. The introduction of phenobarbital into the therapy of epilepsy stimulated the study of related substances, but unless they produced narcosis and prevented chemically-produced convulsions in experimental animals, they were not considered promising.[4] As has been pointed out (1), such investigations have not been particularly fruitful. Although Albertoni (10) long ago showed the influence of bromides on electrically produced convulsions, it remained for Putnam and collaborators to use this method in the search for better drugs.

The anticonvulsant action of diphenyl hydantoin seems to be associated with nervous mechanisms higher than the mesencephalon, since there is no such action in the decerebrate animal. The lack of influence on reflexes in the spinal animal suggests that large doses act through higher nervous mechanisms to cause increased excitability, rigidity, and convulsions.

Diphenyl hydantoin does not prevent convulsions resulting in rabbits from the administration of strychnine and cocaine. It has been reported to prevent metrazol convulsions in mice (11). Conflicting results with metrazol convulsions in man have been reported (12). It is of interest here to recall that phenobarbital was found to be much less effective than barbital and amytal in preventing experimental cocaine intoxication (13).

Diphenyl barbituric acid and diphenylene hydantoin have been shown to have anticonvulsant action, being between one-half and one-fourth as effective as diphenyl hydantoin. Our finding of the inactivity of diphenyl thiohydantoin was unexpected, since barbituric and thiobarbituric acids are similar pharmacologically. Kozelka *et al.* have briefly reported (14) diphenyl thiohydantoin to have anticonvulsant activity.

According to Kozelka and Hine (15), larger amounts of diphenyl hydantoin are excreted in the urine of men than we have found in dogs and rabbits.

## SUMMARY

Diphenyl hydantoin raises the threshold of electrically produced convulsions in cats. This anticonvulsant activity is not seen in decerebrate cats.

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[4] This actually seems to have happened to diphenyl hydantoin. Dox and Thomas (3) remarked that it lacks hypnotic action.

Diphenyl hydantoin does not prevent the convulsions caused by strychnine and cocaine.

Diphenyl hydantoin does not appear as such in the urine of rabbits and dogs receiving large amounts of it.

Diphenylacetyl urea and diphenyl thiohydantoin are devoid of anticonvulsant activity. Diphenyl barbituric acid has from one-fourth to one-half the anticonvulsant activity of diphenyl hydantoin, and an equal acute toxicity. Diphenylene hydantoin has from one-fourth to one-half the anticonvulsant activity, and more than one-half the acute and chronic toxicity, of diphenyl hydantoin. Fluorenone has, on oral administration of large amounts, a slowly developing anticonvulsant activity.

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# THE EXPERIMENTAL PRODUCTION OF PRIMARY OPTIC ATROPHY IN MONKEYS BY ADMINISTRATION OF ORGANIC ARSENICAL COMPOUNDS

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It has long been known that patients afflicted with neurosyphilis or trypanosomiasis have occasionally suffered extensive visual damage or even complete blindness when treated with certain arsenical compounds. The compounds reported as capable of producing visual damage are atoxyl, tryparsamide, stovarsol, and compound No. 190 (*3*-amino-*4*-*beta*-hydroxyethoxy-phenylarsonic acid).

Syphilis of the central nervous system, if left untreated, frequently produces blindness, and trypanosomiasis may possibly do so also; hence it seems quite possible that visual damage observed in treated cases of these two diseases may be due to the disease itself, to the therapeutic agent, or to a combination of factors including others as yet unknown.

Bornemann (1) and Fehr (2) have reported visual damage in patients suffering from *lichen planus rubra* and from pemphigus who were treated with atoxyl. There was a progressive narrowing of the visual fields, particularly on the nasal side, pallor of the disk, and a narrowing of the retinal arteries. Reflexes remained normal and there were no scotomata. In these reports there has been a notable absence of a description of any visible changes in the fundus, hence it may be assumed that such changes were infrequent or very slight when they did occur. Key (3), by the repeated administration of atoxyl to dogs, was able to produce pallor of the optic disk. Unfortunately we cannot be sure that these results were controlled by ophthalmoscopic examination before the administration of the atoxyl or by hemoglobin determinations. None of the animals became blind but histological examination revealed a reduction in the number of ganglion cells and nerve fibers of the retina. Birch-Hirschfeld and Köster (4) felt that the greatest toxic effect of atoxyl was on the peripheral portion of the third optic neuron. Rods and ganglion cells were attacked but the cones were unaffected. Igersheimer (5) is in agreement with Birch-Hirschfeld and Köster as to the point of action of atoxyl. It would seem, therefore, that atoxyl is capable of producing visual damage in patients with an intact and apparently normal central nervous system, as well as in individuals with neurosyphilis or trypanosomiasis, and that certain pathological changes have been produced in the eyes of experimental animals.

Woods and Moore (6) have reported visual damage following the use of tryparsamide in patients suffering from postencephalitic Parkinson's syndrome and from lymphosarcoma. Sloan and Woods (7), in studying this reaction due to tryparsamide, have emphasized the fact that the main early objective evidence is confined to a narrowing of the perimetric fields. In their studies of visual damage due to tryparsamide and of luetic optic atrophy there was reported a greater field contraction for one degree white than for one degree red or blue and, in the early stages, a greater defect for red than for blue. Damage due to tryparsamide was more evident in the nasal than in the temporal field and consequently could usually be distinguished from the optic atrophy of syphilis. The cytological changes directly due to the administration of tryparsamide alone have not been described but the clinical characteristics already mentioned show a striking similarity between visual damage resulting from tryparsamide and that resulting from atoxyl. Pearce (8) found that visual damage occurred only in advanced cases of trypanosomiasis and therefore assumed that the disease might in part be responsible. Woods and Moore pointed out that 22.8% of the patients having general paralysis or tabes dorsalis developed visual disturbances while only 15.2% of the patients having other types of neurosyphilis were similarly affected. The incidence of visual damage in cases of untreated neurosyphilis was near 15% in their series. In spite of the increased incidence apparently due to tryparsamide in cases of neurosyphilis, Reese (9) has pointed out that even visual damage by the disease is not a contraindication to treatment with tryparsamide.

Tatum *et al.* (10) reported contraction of the perimetric fields and blindness in certain cases of neurosyphilis treated with arsenical No. 190.

Sézary and Barbé (11) reported visual disturbances which necessitated a reduction in the dosage of "stovarsol" (acetarsone) in patients affected with neurosyphilis.

From the foregoing review it would seem that atoxyl is capable of producing blindness in patients with a previously normal central nervous system and that tryparsamide, arsenical No. 190 and stovarsol are agents which likewise contribute to visual damage in patients affected with certain diseases of the central nervous system.

It seemed desirable to determine whether or not these arsenicals could produce blindness in experimental animals with an intact and normal central nervous system since such a reaction could be used to test the blinding potentialities of compounds as yet untried in man. Rhesus monkeys were selected since Davis (12) found the monkey eye to resemble the human eye far more closely than does the rabbit eye. Such an anatomical relationship might be expected to be paralleled by a similar reactivity; however, experimental work can only indicate what might happen in man and hence only careful clinical study will reveal what actually does happen.

Gradually increasing doses of the compounds were given intravenously to the monkeys at weekly intervals unless there was a marked loss of weight or other manifestations of toxicity. If toxic symptoms appeared administration was discontinued until the monkey showed a substantial increase in body weight. Symptoms of toxicity consisted of anorexia, weight loss, muscular weakness, incoordination, tremors, at times generalized convulsions, and coma in the terminal stages. Drug administration was continued until the monkeys either died of toxicity or became blind. The development of blindness was followed by an occasional dose of the compound in order to prevent the remote possibility of recovery of visual function. Not all monkeys became blind even though some were given the drugs until fatal poisoning resulted. It is curious to note that those animals which became blind frequently appeared freest from other signs of intoxication, just as tabetics are apt to be free from paresis.

The blindness was of such degree that all observers were satisfied that the monkeys could not see in spite of the fact that the light reflex was usually retained. A blind monkey would not look for his food, but would feel for it. He was apparently not aware of a stick held a few inches from his face and would bump into objects when placed in strange surroundings. Ophthalmoscopic examination of a blind monkey revealed a searching nystagmus. A pallor of the disk usually appeared at 4 to 6 weeks after the monkey became blind. On occasions the pallor was greater on the nasal than on the temporal half of the retina. Narrowing of the retinal vessels was slight and was not of uniform occurrence. A hemoglobin determination done on the blood of three monkeys at the time that pallor of the disk was noted was found to be essentially normal, namely, 10.3, 13 and 14.3 grams per 100 cc., respectively. Histological examination of the eyes of the blind monkeys revealed a decrease in the number of ganglion cells of the retina, a proliferation of glial cells, and a demyelinization of the optic nerve.

Table 1 gives a summary of the monkeys used and the results obtained. It was possible to produce blindness in monkeys with atoxyl, tryparsamide, compound No. 190, stovarsol, and compound No. 266 (*4*-β-(β′-hydroxy)ethoxy ethoxy phenylarsonic acid).

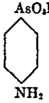
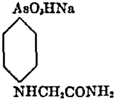
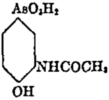
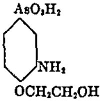
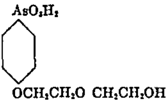
If the monkey is to be an adequate test animal for determining the blinding potentialities of a compound in man, the following considerations must be fulfilled:

*1.* Monkeys must be blinded by all of the compounds producing blindness in man. *2.* Monkeys must not be blinded by compounds which do not produce blindness in man. *3.* The number of test animals must be adequate to insure that the results are not merely chance.

With the exception of compound No. 266, which has not been studied in man, requirement *1* has been adequately fulfilled in the studies reported here. Studies were in progress in order to fulfill requirements *2* and *3*, but owing to the present paucity of test animals the work appears to be unavoidably interrupted for the time being.

The part of the molecule which is responsible for the visual damage has not yet been determined. Young and Loevenhart (13) studied a group of pentavalent arsenical compounds with an amino nitrogen or a substituted amino nitrogen in the benzene ring. It was their opinion that compounds with free or substituted amines in the position para to the arsonic acid would be the ones likely to produce visual damage in man. On the other hand, Sézary and Barbé reported visual damage with stovarsol, which has an acetylamino group in the meta position, and Tatum and associates have reported visual damage with arsenical No. 190, which has an amino nitrogen in the meta and a hydroxyethoxy group in the para position. In our study, compound No. 266, which contains no amino group at all, was found capable of producing the same characteristic visual damage and resulting manifestations that were produced by those compounds containing amino groups. Therefore, at the present time one can say

TABLE 1

| ARSENICAL | MONKEY NUMBER | RESULT | REMARKS | APPROXIMATE BLINDING DOSE *mgm/kgm* |
|---|---|---|---|---|
| Atoxyl  | 1 | Not blind | Died of tox. at 80 mgm./kgm. | |
| | 2 | Not blind | Died of tox. at 120 mgm./kgm. | |
| | 3 | Not blind | Died of tox. at 160 mgm./kgm. | |
| | 13 | Not blind | Died of tox. at 140 mgm./kgm. | |
| | 14 | Not blind | Died of tox. at 70 mgm./kgm. | |
| | 15 | Blind | Killed at 80 mgm./kgm. | 80 |
| Tryparsamide  | 5 | Blind | Died of tox. at 900 mgm./kgm. | 900 |
| | 6 | Not blind | Died of tox. at 900 mgm./kgm. | |
| | 7 | Blind | Killed at 700 mgm./kgm. | 600 |
| | 8 | Blind | Died of tox. at 700 mgm./kgm. | 650 |
| | 9 | Not blind | Died at tox. at 200 mgm./kgm. | |
| | 10 | Blind | Died of tox. at 800 mgm./kgm. | 700 |
| Acetarsone ("Stovarsol")  | 16 | Blind | Killed at 320 mgm./kgm. | 320 |
| | 18 | Blind | Killed at 220 mgm./kgm. | 220 |
| Arsenical No. 190  | 21 | Not blind | Died of tox. and dysentery at 300 mgm./kgm. | |
| | 26 | Not blind | Died of tox. at 450 mgm./kgm. | |
| | 27 | Blind | Died of tox. at 550 mgm./kgm. | 550 |
| Arsenical No. 266  | 24 | Not blind | Died of tox. at 450 mgm./kgm. | |
| | 25 | Not blind | Symptoms at 360 mgm./kgm.<br>Killed after severe toxicity reaction | |
| | 29 | Blind | Killed at 250 mgm./kgm. | 200 |

only that visual damage is not a function of the character of a nitrogen-containing side-chain or of its position or even of its presence.

## SUMMARY

1. Blindness has been produced in monkeys by the administration of the following compounds: atoxyl, tryparsamide, stovarsol, *3*-amino-*4*-beta-hydroxy-ethoxy-phenylarsonic acid (No. 190), and *4*-$\beta$-($\beta'$-hydroxy)ethoxy-ethoxy-phenylarsonic acid (No. 266), the last containing no nitrogen in the molecule.

2. The work herein reported would appear to provide an experimental basis for detecting the blinding potentialities of arsenical compounds for man.

3. Means now become available for a study of prophylactic procedures designed to reduce or eliminate optic atrophy as a sequence of chemotherapy of neurosyphilis.

The writers are grateful to Drs. M. E. Nesbit and F. A. Davis for their interest in making the ophthalmological observations and to Dr. F. A. Davis for permission to include herein a preliminary statement relative to cytological alterations of the retinas and optic nerves. A complete report on these observations will appear in due time.

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# RADIOACTIVE TRACER STUDIES ON ARSENIC INJECTED AS POTASSIUM ARSENITE[1]

## I. EXCRETION AND LOCALIZATION IN TISSUES

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FORWARD. Because potassium arsenite (Fowler's solution) given in small small doses over long periods of time produces temporary improvement in many cases of chronic myeloid leukemia (1),[2] an understanding of the mode of action of this "tissue poison" is highly desirable. The difficulty of chemically estimating small quantities of arsenic, however, has heretofore prevented the elucidation of this problem. But with the recent development of radioactive tracer techniques it became practicable to investigate with some hope of success the course of this element within the body. Therefore in the autumn of 1939, utilizing the facilities of the Massachusetts General Hospital, the Massachusetts Institute of Technology, and of the Harvard Medical School, we began a co-operative research, to determine, if possible, the bodily fate of the inorganic trivalent arsenic atom. Although a preliminary report has already been published (2), the two papers which follow present for the first time the detailed experimental data accumulated from the inception of the work until its discontinuance because of the war.

METHODS. *Preparation of Targets.* Germanium metal is powdered to approximately 100 mesh size and made into a thin paste with a 5% aqueous mixture of Handy Flux.[3] A layer about 0 5 mm. thick is painted on a $\frac{1}{16}''$ sheet of copper previously cleaned with 6 M nitric acid. The copper is cautiously heated from below until the paste is dry and then is heated strongly with a Fisher type burner until the germanium alloys with the copper and forms a smooth melt on the surface. This requires a bright red heat and is readily determined by inspection. When the surface has hardened the target is quenched in water to remove the flux and scale and cleaned in concentrated hydrochloric acid. The back is "tinned" with silver solder and then silver soldered to the probe assembly.

After bombardment by deuterons in the cyclotron, the surface of the target is removed to a depth of about 0 75 mm. with a small end mill in a drill press. This removes all the germanium in the form of a coarse powder which is readily dissolved.

*Chemical Treatment of Targets* (3). An all-glass distilling apparatus utilizing a modified Claissen flask (250 cc.) has been found useful in several radiochemical separations. The coarse powder from the bombarded target is placed in the flask and a standard solution of sodium arsenite added. In order to keep the specific activity as high as possible the quantity of added arsenic must be kept low. The following separation has been made satisfactorily with 0.5 mgm. of arsenic carrier although 2 mgm. is the usual amount used. Below 0.5 mgm. the amount lost becomes an appreciable fraction of the total.

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[1] From the Department of Physics, Massachusetts Institute of Technology, The Medical Services, Massachusetts General Hospital, and the Department of Biochemistry, Harvard Medical School. The expenses of these investigations have been borne by various funds and by contributions of individual donors. Cyclotron time and radioactivity expenses were defrayed in part by the John and Mary R. Markle Foundation, and the salary of our technician by the H. N. C. gift of the Harvard Medical School. For these grants and individual gifts from Mr. and Mrs. G. S. Amory, Mrs. Francis B. Crowinshield, and Mrs. Theodore E. Brown, we are deeply grateful.

[2] The bibliography will be found at the end of the second paper.

[3] Handy and Harmon, 82 Fulton Street, New York, New York.

The target material is treated with 25 cc. of 12 M hydrochloric acid, heated, and dissolved by the dropwise addition of 30% hydrogen peroxide. This operation requires 10 to 20 cc. of hydrogen peroxide and takes about 5 minutes. Germanium is removed as the volatile *tetrachloride by distillation of the hydrochloric acid solution.* Frequent additions of a few drops of hydrogen peroxide minimizes reduction of $As^{+5}$ to $As^{+3}$ by the hydrochloric acid, and loss of arsenic as arsenic trichloride. When solids precipitate from the solution in the flask 10 cc. of 12 M hydrochloric acid are added and the distillation repeated. Glass beads or pieces of tile will reduce bumping of the mixture during distillation.

To reduce the $As^{+5}$ to $As^{+3}$ so arsenic trichloride can be distilled from the solution, 15 cc. of 12 M hydrochloric acid and 10 cc. of 5 M hydrobromic acid are added and the solution distilled into 10 cc. of distilled water cooled in an ice bath. When bromine fumes begin to appear in the distillation flask 10 cc. of 12 M hydrochloric and 5 cc. of 5 M hydrobromic acid should be added and the distillation repeated until bromine fumes again appear. Heating the concentrated hydrochloric acid causes the evolution of a quantity of hydrogen chloride gas which helps to sweep out the small quantity of arsenic trichloride vapor.

Arsenic metal is precipitated from the distillate by adding 1 to 2 grams of solid ammonium hypophosphite and warming on the steam bath for 5 to 10 minutes (4). It is inadvisable to boil the solution or heat it too long as the coagulated precipitate is not readily wetted by the solution and is difficult to transfer to the filter. If coagulation proceeds too far the clumps can be redispersed by adding a few milligrams of "Duponol" or some similar wetting agent.

A 3 cc. porous porcelain micro-filter crucible which has been ground to fit a suitable funnel is very satisfactory for filtering out the precipitated arsenic. It filters rapidly with suction, holds the precipitate completely, is readily dried and can be weighed to check recovery of added arsenic from the separation. Filtration is carried out without allowing air to be sucked through the precipitate until after washing it with 5 to 10 cc. of freshly boiled distilled water and then with acetone. In this way oxidation of the finely divided metal is minimized.

The arsenic metal is oxidized to arsenic acid with one drop of 30% hydrogen peroxide. After filtering into a graduated centrifuge tube, and washing the filter with potassium bicarbonate solution (10 mgm./cc.) the solution is boiled to destroy the peroxide. Twenty-five milligrams of sodium bisulphite are added to reduce arsenate to arsenite. This solution is made isotonic with sodium chloride, sterilized and is ready for use. The final volume is from 5 to 10 cc.

Preparations containing 0.5 to 2 mgm. of As with an activity of 15 to 25 millicuries of 50 hour arsenic ($As^{71}$), and 2 to 3 millicuries of 16 day arsenic ($As^{74}$) have been regularly made by the Massachusetts Institute of Technology cyclotron for this work.

*Measurement Techniques.* In each biological sample the quantity of radioarsenic was measured by means of a Geiger-Müller counter connected to a counting-rate meter (5). This instrument produces a permanent record of the beta ray activity of each sample, and by comparison with a calibrated sample, allows each unknown to be computed. The specific activity of the radioarsenic used and the sensitivity of the counter were such that quantities in many cases as small as $10^{-6}$ and $10^{-5}$ micrograms of arsenic in a given sample could be measured and computed with satisfactory accuracy. In the earlier measurements an end-window-type counter was used. This was a less sensitive instrument because of the smaller solid angle available for the sample; but an improved type, a thin-walled duralumin helium-filled counter, was used in all the later determinations.

All samples were placed on filter paper and wrapped around the counter for measurement. Fluid samples were evaporated on the paper, while tissue samples were mashed into a thin uniform layer and dried. Bone marrow samples were digested before being placed on the filter paper.

For fluid samples, no correction for self-absorption of beta rays by the sample was necessary, since the calibrating standard and fluid samples were prepared in the same way. With tissue samples, an error was introduced due to greater self-absorption by the sample than by the standard. This error was small, however, because of the high energy of beta rays from radioarsenic, and therefore in most instances no correction was made. Errors from this source were found in general to be less than 5%. Skin sample measurements produced errors considerably higher than this, but no attempts were made to correct the results since rough quantitative measurements were sufficient for our purposes.

TABLE 1

*Preliminary studies on rats*

| RAT NO. | DOSAGE OF As | | KILLED AFTER LAST DOSE | ARSENIC CONCENTRATION | | | | |
|---|---|---|---|---|---|---|---|---|
| | Daily | Total | | Whole blood | Liver | Spleen | Kidneys | Brain |
| | *mg./kg.* | *mg /kg* | *days* | *μ/cc* § | *μ/gm.* | *μ/gm.* | *μ/gm.* | *μ/gm.* |
| *A 10♀ | .30 | 1.2 | † | | 1 6 | 2.6 | | |
| *A 6♀ | .52 | 4.1 | 15 | 25 | 2.4 | 5.2 | 3.8 | |
| *A 2♀ | .48 | 5.8 | 22 | 40 | 0 | 2.0 | 0 | |
| B 1♂ | 49 | 4.0 | 6 | 8.3 | .7 | 1.5 | .5 | |
| B 2♂ | .23 | 1 9 | 6 | 5 2 | 3 . | 2.3 | .4 | |
| *B 3♂ | .86 | .86 | 1 | 3 0 | 73 | 1 4 | 1.3 | |
| B 16♂ | 84 | 84 | 1 | 4 5 | 60 | 1.5 | .9 | |
| B 5♂ | .45 | 1 8 | 1 | 18 | 1.2 | 3 3 | .9 | |
| B 20♂ | 68 | 2.7 | 1 | 23 | 2.6 | 6.5 | 1.3 | |
| B 8♂ | 45 | 1 8 | 2 | 15 | 1 1 | 4.5 | .75 | |
| B 23♂ | .69 | 2.8 | 2 | 29 | 2.3 | 9.6 | 2.2 | |
| B 12♂ | .42 | 1 7 | 4 | 14 | 1 1 | 5 1 | .86 | |
| B 21♂ | .62 | 2.5 | 4 | 21 | 1 3 | 6 7 | .63 | |
| B 13♂ | .44 | 1 7 | 6 | 11 | 78 | 2.0 | .50 | |
| B 26♂ | .65 | 2 6 | 6 | 11 | 76 | 2.5 | .42 | |
| B 6♂ | .43 | 1.7 | 8 | 10 | 1 4 | 4.5 | 58 | |
| B 10♂ | .61 | 2.5 | 8 | 9.1 | .59 | 2 8 | .49 | |
| *B 11♂ | 39 | 1.9 | 2 | 14 | 9 | 3 7 | .88 | |
| B 19♂ | .50 | 2.5 | 3 | 13 | 1 1 | 4.0 | 1 3 | |
| B 7♂ | .49 | 2 9‡ | 2 | 19 | 2 1 | 5 6 | 2.0 | .5 |
| B 9♂ | 46 | 2 7‡ | 2 | 19 | 3 1 | 8.1 | 1.4 | .5 |
| C 40♀ | 62 | 3 7‡ | 2 | 19 | 1 5 | 8.2 | 2.7 | .6 |
| C 41♀ | 61 | 3 7‡ | 2 | 35 | 2 7 | 12 | 2.4 | 1.1 |

* Animals had received arsenic previous to radioactive injections.

† Found dead.

‡ Fifth and last dose was double the usual size.

§ In this and the following tables μ signifies microgram (0 001 mgm.).

*Biological Techniques.* The potassium arsenite was in all cases administered as a single daily dose by subcutaneous injection, usually in the form of a 0.1% solution in normal saline. In no instance did a slough or infection occur. The smaller animals were killed by decapitation or etherization, the larger ones (chimpanzees and baboon) by intravenous anaesthesia followed by opening the thorax, and were necropsied immediately. In the case of the human dying of lymphoblastic leukemia, necropsy was performed 2 hours after death.

Throughout the experiments all organ samples were immediately weighed and smeared on filter paper as described above, care being taken that loss by drying during weighing was minimal. Tissues from the three large animals were cut up, placed in stoppered flasks, and immediately frozen with dry ice. Body fluids and excreta were handled so as to prevent evaporation and decomposition.

RESULTS. *Studies on Albino Rats.* Experimental data from 23 rats are summarized in Table 1. The second and third columns show the daily and total doses of arsenic given during the injection period. In the fourth is recorded the number of days elapsing between the final dose and death of the animal, while the fifth column shows the whole-blood arsenic concentration just before death. The remaining columns give organ arsenic concentrations in terms of grams of wet tissue. In every instance the arsenic concentration in the whole blood was considerably greater than in any of the solid organs, and of the latter the spleen invariably gave the highest figures. From the commencement of injections until sacrifice, daily whole blood samples were obtained from each animal and measured. These, without exception, showed a progressive rise of blood arsenic until the day after the final injection, when a slow fall began and continued until the rat was sacrificed.

TABLE 2

*Blood fractionation*

| RAT NO. | PER CENT HEMATOCRIT PLASMA | ARSENIC (μ/CC.) | | | | | PER CENT As IN Rbc. FRACTIONATION | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Plasma | W. blood | Plasma As. per cc. W. blood | Rbc. As. per cc. W. blood | *Per cent* Rbc. As per 1 cc. *blood* | Lipoid | Heme | Globin |
| B 11 | a) 54 | .47 | 15 | .25 | 14.3 | 98 | | | |
| | b) 55 | .68 | | .37 | 14.2 | 97 | | | |
| Avg. | 54.5 | .575 | 15 | .31 | 14.3 | 98 | | | |
| B 19 | a) 65 | .60* | 15 | .38 | 14.5 | 97 | | | |
| | b) 67 | | | .40‡ | 14.5‡ | 97‡ | | | |
| Avg. | 66 | | 15 | .39 | 14.5 | 97 | | | |
| B 7†<br>B 9<br>C 40<br>C 41 | 70 | .64 | 21 | .45 | 21 | 98 | 0 | 34 | 62 |

* a and b samples pooled.
† Pooled.
‡ Calculated.

When it was found that a large proportion of the arsenic in the rat was in the blood, a determination of the concentration in plasma and erythrocytes was indicated. Blood from rats B 11 and B 19 was obtained and prevented from clotting with sodium oxalate, a standard amount per cc. of blood being employed to avoid variations in cell volume. Oxalate was chosen as more convenient, since it had previously been shown that oxalated and heparinized blood from the same animal showed no differences in the arsenic content of the plasma.

Table 2 shows that over 95% of the whole-blood arsenic is localized within the erythrocytes, a finding completely unanticipated. When the blood was laked with 0.1% $Na_2CO_3$ and dialyzed through cellophane, no arsenic could be demonstrated in the dialysate, the concentration within the sac remaining virtually unchanged. This indicates that the arsenic was firmly attached to a large molecule. Blood was therefore obtained from 4 animals, pooled, and after removal of the plasma, the cells were laked and the lipoids extracted with toluene. This fraction showed no arsenic, whereas the globin and acid-acetone soluble heme fractions from the same sample contained almost all of the erythrocyte arsenic.

TABLE 3

*Comparative studies on arsenized and unpoisoned rats*

| RAT NO. | DOSAGE OF As | | KILLED AFTER LAST DOSE | ARSENIC CONCENTRATION | | | |
|---|---|---|---|---|---|---|---|
| | Daily | Total | | Whole blood | Liver | Spleen | Kidneys |
| | *mg /kg.* | *mg /kg.* | *days* | *μ/cc.* | *μ/gm.* | *μ/gm.* | *μ/gm.* |
| *B 4 ♂ | .54 | 2.2 | 3 | 19 | 1 1 | 4.2 | 1.0 |
| B 24 ♂ | .51 | 2.0 | 3 | 16 | 1.3 | 6.1 | 1.1 |
| *B 17 ♂ | .60 | 2.4 | 5 | 22 | 1.9 | 6.9 | .7 |
| B 22 ♂ | .66 | 2.6 | 5 | 14 | 1.1 | 6.9 | .3 |

* Animals had received arsenic previous to radioactive injections.

TABLE 4

*Studies on splenectomized rats*

| RAT NO. | DOSAGE OF As | | KILLED AFTER LAST DOSE | ARSENIC CONCENTRATION | | | |
|---|---|---|---|---|---|---|---|
| | Daily | Total | | Whole blood | Liver | Spleen | Kidneys |
| | *mg /kg.* | *mg./kg.* | *days* | *μ/cc.* | *μ/gm.* | *μ/gm.* | *μ/gm.* |
| *C 39 ♀ | .68 | 2 7 | 1 | 19.0 | 1.4 | 5 3 | .96 |
| C 30 ♀ | .67 | 2.7 | 1 | 18 | 1.6 | | 2.1 |
| C 34 ♀ | .66 | 2.6 | 2 | 17 | 1.2 | | 1.6 |
| C 35 ♀ | .71 | 2.8 | 4 | 28 | 1 5 | | 1.2 |
| C 36 ♀ | .67 | 2 7 | 6 | 22 | 1.2 | | 1.6 |
| C 38 ♀ | .79 | 3.2 | 8 | 8.2 | .6 | | .9 |

* Control.

To determine whether or not previous arsenic administration would change the distribution of arsenic injected at a later time, two animals were given nonradioactive arsenic over considerable periods of time and then radioactive tracer arsenic. The results in Table 3 show no difference between such animals and fresh controls. Similarly, as Table 4 indicates, splenectomy prior to administration of arsenic failed to influence its distribution.

Investigation of a high post-operative mortality in the splenectomized animals before injections could be begun, revealed that all of our rats were infected with *Bartonella muris*. As this organism is killed by certain arsenicals the question arose whether the arsenic within the erythrocytes was not to a certain extent bound to proteins of this organism. Therefore a group of uninfected rats was obtained and the earlier type of experiment was repeated. The data in Table 5 show that Bartonella-free animals have the same pattern of arsenic distribution as infected rats.

While these rats, living in the same cage, were undergoing daily injection the urine and feces of all four were carefully collected. Table 6 shows the total arsenic excreted each day by all four animals, beginning on the day after the first, and ending on the day after the last injection. From that day onward, however, no arsenic excretion could be detected,—an observation in contrast to that made later on human subjects.

TABLE 5

*Studies on bartonella free rats*

| RAT NO. | DOSAGE OF As | | KILLED AFTER LAST DOSE | ARSENIC CONCENTRATION | | | | |
|---|---|---|---|---|---|---|---|---|
| | Daily | Total | | Whole blood | Liver | Spleen | Kidneys | Brain |
| | *mg./kg.* | *mg./kg.* | *days* | *μ/cc.* | *μ/gm.* | *μ/gm.* | *μ/gm.* | *μ/gm.* |
| D 52 ♂ | .64 | 2.5 | 1 | 16 | 2.2 | 6.4 | 3.1 | .6 |
| D 53 ♂ | .55 | 2.2 | 2 | 19 | 3.8 | 5.5 | 4.3 | .8 |
| D 54 ♂ | .57 | 2.3 | 4 | 16 | 1.8 | 4.7 | 3.6 | .5 |
| D 55 ♂ | .59 | 2.3 | 6 | 17 | 2.8 | 4.3 | 3.1 | .5 |

TABLE 6

*Daily excretion of arsenic during injection period**

| | DAY | | | | TOTAL |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | |
| Total arsenic............ | 50μ | 150μ | 285μ | 370μ | 850μ |

* Pooled urine and feces—rats nos. 52, 53, 54, and 55.

*Studies on Guinea Pigs.* Table 7 shows the distribution of arsenic in the organs and in certain body fluids of 14 guinea pigs. In contrast to rats, these animals, and all other animals and humans studied, showed extremely small amounts in the blood. At this point it was thought that if arsenic improves leukemia by direct action on tumor cells, large amounts should be found in rapidly growing tissues such as the testes. This assumption was found to be untrue. On the contrary, the concentration was quite high in the epididymis and liver, neither of which normally has high mitotic activity. Again in contrast to rats, the liver arsenic of guinea pigs was much higher as a rule than that of the spleen. The high concentration found in the tissues of animal #6 is probably accounted for by the fact that it was found dead of pneumonia the morning after the last injection; the renal output of arsenic had in all likelihood been partially arrested by the toxemia of the disease.

TABLE 7

*Studies on guinea pigs*

| | ANIMAL NO. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1♂ | 2♂ | 6♂ | 3♂ | 4♂ | 5♂ | 9♂ | 10♂ | 11♂ | 12♂ | 13♂ | 14♂ | 15♂ | 20♂ |
| Daily As (*mg /kg.*). | 1.6 | .87 | .24 | .25 | .25 | .23 | .82 | .80 | .77 | .77 | .82 | 1.56 | 1.3 | .27 |
| Total As (*mg./kg.*). | 4.95 | 3.5 | .97 | 1.0 | 1.0 | .94 | 3.3 | 3.2 | 3.1 | 3.1 | 3.3 | 1.56 | 1.3 | 1.05 |
| Killed after last dose | 2 | 2 | 1* | 2 | 4 | 6 | 1 | 2 | 4 | 6 | 8 | 1 | 1 | 15† |
| Whole blood (*μ/cc.*). | .05 | 0 | .11 | .04 | 0 | 0 | .05 | .05 | ‡ | ‡ | ‡ | 076 | .06 | .029 |
| Liver (*μ/ gm.*)... | .61 | 1.8 | 2.0 | .63 | .075 | .02 | .85 | .3 | .36 | .06 | .1 | .71 | .52§ | .31 |
| Spleen (*μ/ gm.*) . | .19 | 1 2 | .58 | .20 | .04 | .01 | .26 | .12 | .38 | .06 | .02 | .16 | .16 | .067 |
| Kidney (*μ/ gm.*) | .57 | .5 | 3.22 | .32 | .13 | 04 | .63 | .45 | .35 | .13 | .10 | .65 | .58 | .29 |
| Brain (*μ/ gm.*) . | .23 | .5 | .14 | .29 | 06 | .04 | .20 | .15 | .17 | .08 | .17 | .09 | .13 | .062 |
| Bone marrow (*μ/ gm.*) | .19 | | .45 | .12 | 02 | 0 | .36 | .10 | .15 | 0 | .016 | .24 | .16 | .065 |
| Lung (*μ/gm.*) | | | .72¶ | .19 | 03 | 0 | .27 | .13 | .10 | .026 | .025 | .29 | .17 | .12 |
| Muscles (*μ/ gm.*). . | | | .33 | .19 | 04 | .015 | .25 | .15 | .16 | .05 | .10 | .17 | .14 | .087 |
| Thyroid (*μ/ gm.*) | | | | | | | | .09 | .10 | 0 | 0 | .08 | .10 | .085 |
| Testes (*μ/ gm.*). . | | | | | | | | | | | | .09 | .05 | .038 |
| Epididymis (*μ/gm.*). | | | | | | | | | | | | .75 | .47 | .71 |
| Bladder urine (*μ/ cc.*) . . | | | | | | | | | | | | .8 | .25 | .79 |
| Bile (*μ/cc.*) . | | | | | | | | | | | | | | .13 |

* Found dead.

† Hours.

‡ Too low to read.

§ Tumor of liver, 0.84.

¶ Diseased lung, 0 50.

*Studies on Rabbits.* Data on rabbits in Tables 8 and 9 do not differ essentially from those obtained in guinea pigs. Here, too, we found relatively high arsenic concentrations in the epididymes, and only a moderate concentration in the

## TABLE 8

*Studies on male rabbits*

| | ANIMAL NO. | | | | |
|---|---|---|---|---|---|
| | *312 | *348 | 1 | 2 | 7 |
| Daily As (*mg./kg.*) | .31 | .24 | .8 | .85 | .91 |
| Total As (*mg./kg.*) | .62 | 1.2 | 3.2 | 3.4 | .91 |
| Killed after last dose (*days*) | 1† | 2 | 1 | 4 | 1 |
| Whole blood (*μ/cc.*) | 0 | .17 | .05 | .05 | .04 |
| Liver (*μ/gm.*) | .10 | .12 | .73 | .40 | .24 |
| Spleen (*μ/gm.*) | .07 | .13 | .29 | .17 | .09 |
| Kidney (*μ/gm.*) | .11 | .40 | .88 | .82 | .30 |
| Brain (*μ/gm.*) | .02 | .04 | .13 | .072 | .034 |
| Bone marrow (*μ/gm.*) | | | .23 | .10 | .054 |
| Lung (*μ/gm.*) | | | .59 | .20 | .26 |
| Muscle (*μ/gm.*) | | | .33 | .32 | .074 |
| Thyroid (*μ/gm.*) | | | .18 | .10 | .11 |
| Testes (*μ/gm.*) | | | .16 | .12 | .052 |
| Epididymis (*μ/gm.*) | | | .38 | .87 | .13 |
| Bile (*μ/cc.*) | | | .32 | | .15 |
| Bladder urine (*μ/cc.*) | | | | | 2.0 |

* Liver heavily infected with coccidia.

† Found dead.

## TABLE 9

*Studies on female rabbits*

| | VIRGIN RABBITS | | | RABBITS WITH POSITIVE A. Z. TESTS | |
|---|---|---|---|---|---|
| | Animal no. | | | | |
| | *4 | 5 | 8 | 3 | 6 |
| Daily As (*mg./kg.*) | .96 | .99 | .98 | 1.1 | .84 |
| Total As (*mg./kg.*) | .96 | .99 | 3.91 | 1.1 | 3.3 |
| Killed after last dose (*days*) | 1 | 1 | 1 | 1 | 1 |
| Whole blood (*μ/cc.*) | 0 | .084 | .069 | .13 | .05 |
| Liver (*μ/gm.*) | .034 | .39 | .63 | .14 | .66 |
| Spleen (*μ/gm.*) | .009 | .13 | .22 | .09 | .18 |
| Kidney (*μ/gm.*) | .01 | .49 | .83 | .25 | .89 |
| Brain (*μ/gm.*) | .005 | .057 | .10 | .044 | .10 |
| Bone marrow (*μ/gm.*) | .005 | .15 | .11 | .095 | .10 |
| Lung (*μ/gm.*) | .05 | .62 | .78 | .28 | .75 |
| Muscle (*μ/gm.*) | 0 | .25 | .20 | .12 | .19 |
| Thyroid (*μ/gm.*) | .02 | .15 | .23 | .10 | .19 |
| Ovaries (*μ/gm.*) | 0 | .08 | .14 | .10 | .15 |
| Uterus (*μ/gm.*) | .01 | .12 | .11 | .17 | .18 |
| Bile (*μ/cc.*) | 0 | .34 | .64 | .54 | .06 |
| Bladder urine (*μ/cc.*) | .25 | 2.75 | 8.3 | 5.1 | None |

* Absorption from injection very low.

testes. Female rabbits, virgins and those with active follicle growth (produced by using animals showing a positive Ascheim-Zondek test) revealed no particular differences in concentration in the ovarian tissue. Therefore we must conclude that there is no evidence that arsenic tends to collect in rapidly growing tissues.

*Studies on Human Subjects.* Table 10 contains the results of studies on excretion of arsenic in two healthy human subjects. At a glance it is seen that practically none of the injected dose is excreted via the intestinal tract, and in contrast to rats, the human subject continues to excrete daily lessening amounts for some time after the last injection. In both subjects no arsenic could be measured in the whole blood at any time.

By using radioarsenic of high specific activity it was possible to obtain measurements of blood arsenic as well as further data on urine and stool excretion. These data are shown in Tables 11 and 12. The metal appears in the blood stream shortly after injection, but by the following day has all but disappeared. It continues, however, to appear in small amounts in the urine for some time afterward.

TABLE 10

*Excretion studies in humans—normals*

| DAY | O. H. P. | | | M. B. | | |
|---|---|---|---|---|---|---|
| | Dosage As inj. | Total daily excretion (μ) | | Dosage As inj. | Total daily excretion (μ) | |
| | | Urine | Stool | | Urine | Stool |
| | *mg.* | | | *mg.* | | |
| 1 | 1.5 | | | 1.5 | | |
| 2 | 1 5 | 840 | .17 | 1.5 | 655 | |
| 3 | 1.5 | 550 | | 1.5 | 600 | 1.0 |
| 4 | 1.5 | 750 | 1.1 | 1.5 | 690 | .02 |
| 5 | | 690 | 1.3 | | 250 | .01 |
| 6 | | 140 | | | 85 | |
| 7 | | 68 | | | 45 | |
| 8 | | 42 | | | 35 | |
| 9 | | 35 | | | 45 | |
| 10 | | | | | 20 | |

Table 13 shows the concentration of arsenic in the bone marrow of two patients with leukemia. Biopsy was performed on the day following the injection. In one patient the marrow arsenic was two times that of the blood; in the other, about one and a half times as much. Both patients had high leucocyte counts at the time of biopsy. It was therefore possible, by measuring the whole blood and plasma arsenic, and by making a leucocyte cream, counting the number of leucocytes and measuring the arsenic concentration of the cream, to calculate the amount of arsenic per erythrocyte and per leucocyte. In both patients the amount per leucocyte was roughly ten times that per erythrocyte. But correcting for volume of the cells, this figures reduced to about 3 times as much arsenic in 1 cc. of leucocytes as in an equal volume of erythrocytes.

## TABLE 11

*Excretion studies in humans—leukemias*

| DAY | F. R. | | | | W. L. | | | |
|---|---|---|---|---|---|---|---|---|
| | Dosage As inj. | Blood As | Total daily excretion (μ) | | Dosage As inj. | Blood As | Total daily excretion (μ) | |
| | | | Urine | Stool | | | Urine | Stool |
| | *mg.* | *μ/cc.* | | | *mg.* | *μ/cc.* | | |
| 1 | 1.37 | | | | 4.0 | | | |
| 2 | 1.37 | <.01 | 290 | 0 | 4.0 | .014 | 813 | No stool |
| 3 | 1.37 | <.006 | 405 | 3.8 | 4.0 | 0 | 1980 | No stool |
| 4 | 1.37 | .07 | 420 | 2.5 | 4.0 | .007 | 2130 | No stool |
| 5 | | <.006 | 460 | 2.1 | 4.0 | 0 | 2300 | 83 |
| 6 | | <.006 | 190 | 8.5 | 4.0 | .01 | 1150 | No stool |
| 7 | | <.006 | 150 | 2.7 | 4.0 | .11 | 2120 | No stool |
| 8 | | <.006 | 90 | 1.9 | 4.0 | 0 | 1880 | No stool |
| 9 | | <.006 | 45 | 2.3 | 4.0 | .01 | 900 | No stool |
| 10 | | <.006 | 45 | 1.5 | 4.0 | .01 | 1490 | 330 |
| 11 | | <.006 | 40 | No stool | | .01 | 985 | No stool |
| 12 | | <.006 | 17 | 2.4 | | .01 | 565 | No stool |
| 13 | | <.006 | 16 | 1.6 | | .01 | 400 | No stool |
| 14 | | <.006 | 6 | 1.5 | | | 430 | No stool |

## TABLE 12

*Excretion studies on humans—leukemias*

M. B.

| DAY | DOSAGE As INJ. | BLOOD As | URINE As | DAY | DOSAGE As INJ. | BLOOD As | URINE As |
|---|---|---|---|---|---|---|---|
| | *mg.* | *μ/cc.* | *μ total* | | *mg.* | *μ/cc.* | *μ total* |
| 1 | 1.94 | .055 | | 22 | | .002 | 23 |
| 2 | | 0 | 990 | 23 | | | 28 |
| 3 | | 0 | 247 | 24 | | | 28 |
| 4 | | 0 | 100 | 25 | | 0 | 16 |
| 5 | | 0 | 205 | 26 | | | 15 |
| | | | | 27 | | 0 | 27 |
| 11 | 1.57 | .052 1 hr. | | 28 | | | 10 |
| | | .040 2 hr. | | 29 | | | 8 |
| | | .027 3 hr. | | | | | |
| 12 | | .013 | 122 | 30 | .825 | 0 | 10 |
| 13 | | | 124 | 31 | | 0 | 337 |
| 14 | | | 117 | 32 | | | 48 |
| 15 | | | 73 | 33 | | 0 | 37 |
| 16 | | | 13 | 34 | | | 11 |
| | | | | 35 | | | 22 |
| 17 | 2.18 | .15 1½ hr. | 67 | 36 | | | 13.5 |
| | | .10 3½ hr. | | 37 | | 0 | 7.4 |
| 18 | | .015 | 350 | 38 | | | 13 |
| 19 | | .010 | 141 | 39 | | | 5.6 |
| 20 | | .006 | 94 | 40 | | | 5.0 |
| 21 | | .003 | 45 | | | | |

TABLE 13

*Bone marrow biopsies*

| DAY | DOSAGE As INJ. | BLOOD As | URINE As | BONE MARROW |
|---|---|---|---|---|
| **M. C.** | | | | |
| | *mg* | *μ/cc.* | *μ total* | *μ/gm* |
| 1 | 2.0 | | | |
| 2 | | .0075 | 1060 | .0126 |
| 3 | | .0037 | 100 | |
| 4 | | | 77 | |
| 5 | | | 37 | |
| 6 | | | 30 | |
| 7 | | | 17 | |
| 8 | | | | |
| **O. V.** | | | | |
| 1 | 2.0 | | | |
| 2 | | .013 | 700 | .028 |
| 3 | | 0 | 112 | |
| 4 | | | 89 | |
| 5 | | | 77 | |
| 6 | | | 57 | |
| 7 | | | 44 | |
| 8 | | | 24 | |

TABLE 14

*Lymphatic leukemia—organs*

W. L.

| ORGAN | As |
|---|---|
| | *μ/gm.* |
| Liver | .4 |
| Spleen | .2 |
| Kidney | .54 |
| Cerebrum | .15 |
| Cerebellum | .14 |
| Lung | .06 |
| Pect. muscle | .21 |
| Heart muscle | .10 |
| Heart thrombus | .13 |
| Pancreas | .17 |
| Adrenals | .10 |
| Lymph node | .10 |
| Testes | .14 |
| Vert. marrow | .12 |
| Fem. marrow | 0 |

A patient with lymphoblastic leukemia (W. L. in Table 11) died four days after the last injection. Table 14 shows the arsenic concentration of the organs. The distribution in this case was quite similar to that found in guinea pigs and rabbits. In the latter animals as well as in this case, it should be noted that the largest total amount of arsenic in the body was found in the skeletal muscle, since this tissue makes up approximately 40% of the body weight.

Two patients who were to have pneumoencephalography were injected about 24 hours beforehand. When the spinal fluid was drained off, the blood arsenic was measured and spinal fluid samples taken. As shown in Table 15, although the blood could be easily quantitated, no arsenic could be detected in the spinal fluid. The latter was normal in all other respects. It therefore seems reasonable to assume that the normal choroid plexus is relatively impervious to arsenic passing through it from the blood into the spinal fluid. In regard to permeability to arsenic in the other direction, i.e., from spinal fluid to blood stream, it was not possible to obtain data.

TABLE 15

*Spinal fluid studies—humans*

| DAY | DOSAGE As INJ. | BLOOD As | URINE As | SP. FLUID As |
|---|---|---|---|---|
| R. B. | | | | |
| | *mg.* | *μ/cc.* | *μ total* | *μ/cc.* |
| 1 | .73 | | | |
| 2 | | .006 | 520 | 0 |
| 3 | | | | |
| J. C. | | | | |
| 1 | 1.65 | | | |
| 2 | | .009 | 1020 | 0 |
| 3 | | | 60 | |

TABLE 16

*Higher apes—body fluids*

| | ANIMAL NO. | | |
|---|---|---|---|
| | Chimp. 86 ♀ | Chimp. 112 ♂ | Baboon 1702 ♂ |
| Daily As (*mg./kg.*) | .065 | .057 | .14 |
| Total As (*mg./kg.*) | .065 | .057 | .14 |
| Killed after last dose (*days*) | 1 | 1 | 7 |
| Whole blood (*μ/cc.*) | .0089 | .0057 | .0024 |
| Plasma (*μ/cc.*) | .0012 | .00081 | .0010 |
| Serum (*μ/cc.*) | .00094 | | |
| Bile (*μ/cc.*) | .077 | .017 | .015 |
| Urine (*μ/cc.*) | * | .088 | .045 |
| Sp. fluid (*μ/cc.*) | .0025 | .00018 | .0014 |

* Bladder empty.

*Studies on Higher Apes.* In regard to the last 3 animals a word of explanation is needed. Chimpanzee #86 was injected 24 hours prior to beginning anesthesia for a protracted physiological experiment on its brain. Since the brain was exposed for 10 hours before death took place, the experiment itself influenced the arsenic distribution only because of dehydration and blood loss. Otherwise the animal was healthy. Chimpanzee #112 was quite sick when first injected and at necropsy the following day showed extensive miliary tuberculosis of the lungs, but very little of the abdominal organs. Baboon #1702 was a healthy vicious animal, although at necropsy it showed heavy infestation of the large bowel with a trematode (*Watsonius watsoni*). There is no reason to suppose that this infestation influenced the arsenic distribution to any extent. All of these animals received a very small dose per kilo of weight, but the specific activity was so high that quantitations could be carried out with a high degree of accuracy.

TABLE 17

*Higher apes—organs*

| ORGAN | CHIMP. 86 ♀ As | CHIMP. 112 ♂ As | BABOON 1702 ♂ As |
|---|---|---|---|
| | *μ/gm.* | *μ/gm.* | *μ/gm* |
| Liver | .26 | .11 | .052 |
| Spleen | .065 | .054 | .023 |
| Kidney | .21 | .082 | .080 |
| Cervical cord | .0039 | | .0033 |
| Cerebellum | | .0032 | .0085 |
| Cerebrum | | .0026 | .0039 |
| Lung | .038 | .015 | .011 |
| Muscle | .021 | .011 | .013 |
| Thyroid | .012 | .013 | .029 |
| Thymus | .060 | | |
| Ovaries | .024 | | |
| Uterus | .031 | | |
| Epididymis | | .004 | .075 |
| Testes | | .012 | .010 |
| Heart | .084 | .030 | .012 |
| Pancreas | .068 | .032 | .014 |
| Pituitary | .032 | .028 | .017 |
| Adrenals | .023 | .021 | .006 |
| Cardiac stomach | .036 | | .011 |
| Pylorus | .033 | | .028 |
| Bone marrow | .023 | .022 | .010 |
| Seminal vesicle | | | .027 |
| Prostate | | | .023 |
| Parasites | | | .10 |
| Daily As (*mg./kg.*) | .065 | .057 | .14 |
| Total As (*mg./kg.*) | .065 | .057 | .14 |
| Killed after last dose (*days*) | 1 | 1 | 7 |

Table 16 shows the arsenic concentration in the whole-blood, plasma, bile, urine, and spinal fluid at necropsy of three higher apes. In each of these animals the small amount in the blood appeared to be chiefly in the erythrocytes. The bile showed a fairly high concentration, and although the feces of these animals were not examined, if they should have shown a low amount, such as was noted in humans, it would indicate reabsorption in the intestinal tract. A relatively low concentration was observed in the spinal fluid, but not as low in relation to the blood level as was found in the human subjects. Insufficient data make it impossible to assign a reason for the different whole-blood : plasma ratio noted in the baboon as compared to that found in the two chimpanzees. The baboon and chimpanzee #86 showed spinal fluid arsenic concentrations greater than that of the plasma, whereas the spinal fluid arsenic of chimpanzee #112 was much lower than that of the plasma. But since the latter animal was starved, dehydrated, and very sick, the data obtained on it must be viewed with some suspicion.

In the last table (Table 17) the arsenic concentration in the organs of the three higher apes is recorded. The baboon showed a high concentration in the epididymis, but in the tubercular chimpanzee it was relatively low—the first instance of this encountered in any male animal. The high concentration in the intestinal parasites was of some academic interest. Again it should be noted that the largest total amount of arsenic was found in the skeletal muscle.

## *CONCLUSIONS*

1. In the rat arsenic injected as potassium arsenite is concentrated in the erythrocytes and appears to be bound to the hemoglobin molecule.
2. In other animals and in humans arsenic is more widely distributed, but the largest total amount is stored in the skeletal muscles.
3. Excretion in humans and possibly in most animals is almost entirely via the kidneys.
4. Arsenic does not pass from the blood into the spinal fluid in detectable amounts in humans, but in apes some does get through.
5. There is no evidence that arsenic accumulates in rapidly growing tissues.
6. Of the small amount found in the whole blood of two leukemic patients, there was between three and six times as much in 1 cc. of leucocytes as in the plasma; and between one-half to an equal amount distributed between erythrocytes and plasma. Per cell, the leucocyte averages ten times the amount of arsenic as the erythrocyte.
7. Tissue concentrations of arsenic have been measured in rats, rabbits, guinea pigs, chimpanzees, a baboon, and one human dying of lymphoblastic leukemia.

# RADIOACTIVE TRACER STUDIES ON ARSENIC INJECTED AS POTASSIUM ARSENITE

## II. CHEMICAL DISTRIBUTION IN TISSUES

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The preceding paper has shown that small amounts of arsenite tagged with $As^{74}$, when injected into the body, become distributed in the various tissues at concentrations more or less characteristic for each tissue. These arsenic concentrations, in general, are much greater in the tissues than in the serum. This suggested that a non-diffusible combination of the injected arsenic is formed within the cells. The present paper presents the results of our investigations of the manner in which arsenic is held by the tissues after its administration.

Because of its extreme sensitivity the radioactive tracer method has proved of great advantage in this work. For example, following the administration of a non-toxic amount of arsenite containing $As^{74}$, a sample of liver had so much radioactivity that only 3 mgm. of tissue, or an equivalent fraction of a larger tissue sample, were required for analysis. This amount of tissue contained only $10^{-6}$ mgm. of arsenic.

Voegtlin, Dyer and Leonard (6) suggested that the sulfhydryl groups in reduced glutathione and in proteins combine with trivalent arsenic, and Rosenthal (7) observed the *in vitro* combination of arsenoxide (*3*-amino-*4*-hydroxyphenyl arsenious oxide) with proteins in parallel with their content of free sulfhydryl groups. However, it was not possible for them to demonstrate that arsenic combined exclusively with sulfhydryl groups. The further possibility exists that arsenic might replace phosphorus, since arsenate will apparently substitute for phosphate in at least one step in the glycolytic cycle, and it has been reported that liver nucleoproteins will take up arsenic (8). To investigate the relationship of arsenic to phosphorus was one object of our work.

The first step in tracing the arsenic was to divide the tissues into acid-soluble, lipid, and protein fractions. The acid-soluble fraction should contain (*a*) inorganic arsenic, (*b*) arsenic which might have replaced phosphorus in the acid-soluble organic phosphorus compounds, and (*c*) any arsenic in combination with reduced glutathione. Following this general fractionation, the protein portion, which was found to contain the bulk of the arsenic, was further fractionated, chiefly with ammonium sulfate. Analyses for phosphorus as well as for arsenic were made on the various fractions to see if any correlation could be observed in the distribution of these two elements.

Although the arsenic did not prove to be confined exclusively to any group of proteins, definite differences were observed in the amount of arsenic in different protein fractions.

METHODS. The tissue acid-extracts were prepared with 0.75 M nitric acid. The lipids were extracted from the acid-insoluble residue with alcohol and ether. The lipid-free residue was taken as the "protein" fraction although it obviously would contain some glycogen. These fractions were then digested with sulfuric and fuming nitric acids, a definite volume of 3% sodium bicarbonate was added to each sample, and aliquots were taken for measuring the radioactivity and phosphate (9).

For the protein fractionation, the tissue was minced and then thoroughly broken up in a Waring blender with ice cold 1% sodium chloride solution at pH 7.4. The portion of the tissue in solution after this treatment was used for fractionation of the proteins with different concentrations of ammonium sulfate (6°C.).

In order to precipitate each fraction a number of times with ammonium sulfate without too much loss, a scheme of precipitation was used which was analogous to the procedure employed in making repeated fractional distillations. After making the initial successive precipitations (*1a*, *1b*, etc.), the fraction *1a*, insoluble in the lowest salt concentration (0.6 M $(NH_4)_2SO_4$), was dissolved insofar as possible in water, centrifuged, and precipitate *2a* obtained by adding to the separated supernatant fluid a sufficient quantity of ammonium sulfate to bring the salt concentration to 0.6 M once more. At this point instead of discarding the resultant supernatant, it was used to redissolve precipitate *1b*, previously obtained, with the next higher salt concentration (1.2 M). Any of this precipitate which failed to redissolve in the 0.6 M ammonium sulfate was combined with precipitate *2a* for succeeding reprecipitations. Meanwhile precipitate *2b* was obtained by adding ammonium sulfate to the supernatant liquid to obtain a concentration of 1.2 M. This general procedure was followed with each of the other fractions obtained with increasing ammonium sulfate concentrations. By thus retaining all of the supernatant fluids, the only protein lost was that which failed to dissolve in water at one end of the scale, and that which failed to precipitate with the highest ammonium sulfate concentration (3.6 M) at the other. Each fraction was precipitated four times, following which it was washed with 10% trichloracetic acid, then with a 1:3 alcohol-ether mixture, dried, weighed, wet-ashed, and finally analyzed for phosphorus and arsenic.

In addition several samples of nucleoprotein were prepared. The method of Greenstein (10) was followed initially, but since this involves bringing the preparation to pH 10.5, and since at this pH arsenic was found to be partially split from the proteins, the procedure was modified to avoid pH's above 8.5. As a result, the yield was decreased, but the phosphorus content was increased from about 1% to over 4%.

Results. In Table 1 is recorded the distribution of arsenic and phosphorus in the acid-soluble, lipid, and "protein" fractions obtained from five tissues and the serum of a guinea pig, and from the liver and kidney of a chimpanzee and of a baboon. The guinea pig had been injected 8 times with arsenite over a period of 4 days, the chimpanzee had received a single injection 24 hours previously, while the baboon had been injected one week before the samples were obtained. With the exception of the serum, the protein fraction appeared to contain the bulk of the arsenic, with a variable amount in the acid-soluble fraction and a negligible amount in the lipid fraction.

Not all of the acid-soluble arsenic was dialyzable through a cellophane membrane. It may be mentioned at this point that in contrast to the findings in regard to the tissues, in urine, serum, and bile practically all of the arsenic was found to dialyze readily through a cellophane membrane.

In the case of liver and kidney, the tissue proteins were further fractionated to see if the arsenic was concentrated in any particular group. Some typical data for the liver of the same chimpanzee mentioned above are shown in Table Per gram of protein there was three times as much arsenic in the less soluble pr

TABLE 1

*Distribution of arsenic and phosphorus in fractions of various tissues (values reported per kg. of fresh tissue)*

| | ACID-SOLUBLE | | | LIPID | | | PROTEIN | | |
|---|---|---|---|---|---|---|---|---|---|
| | As $10^{-7}$ mol | P $10^{-3}$ mol | As/P x$10^4$ | As $10^{-7}$ mol | P $10^{-3}$ mol | As/P x$10^4$ | As $10^{-7}$ mol | P $10^{-3}$ mol | As/P x$10^4$ |
| **Guinea pig** | | | | | | | | | |
| Liver | 3.5 | 36.1 | .097 | .5 | 37.0 | .01 | 32.6 | 31.4 | 1 04 |
| Kidney | 2.7 | 26 2 | .103 | .7 | 26.6 | .03 | 29.0 | 28.4 | 1.02 |
| Spleen | $<.3$ | 39.5 | $<.01$ | $<.3$ | 17.5 | $<.02$ | 8.4 | 50.8 | .14 |
| Brain | .4 | 24.9 | .016 | .1 | 59.7 | .002 | 11.7 | 15.5 | .75 |
| Muscle | .2 | 59.4 | .004 | .2 | 10.9 | .02 | 9.1 | 4.8 | 1.90 |
| Serum | .8 | .70 | 1.1 | .1 | .32 | .3 | .06 | .06 | 1.0 |
| **Chimpanzee #112** | | | | | | | | | |
| Liver | 4.0 | 32.6 | .12 | .1 | 24.8 | .006 | 10.6 | 25.3 | .42 |
| Kidney | 3.7 | 38.3 | .10 | .1 | 23.0 | .004 | 5.9 | 15.1 | .39 |
| **Baboon** | | | | | | | | | |
| Liver | .9 | 34.0 | .03 | .4 | | | 5.7 | 23.4 | .24 |
| Kidney | 1.9 | | | | | | 8.1 | 26.8 | .30 |

TABLE 2

*Distribution of arsenic and phosphorus in protein fractions of the liver*

| PROTEIN FRACTION | PER CENT OF TOTAL PROTEIN | As $10^{-7}$ MOLS PER KG PROTEIN | P $10^{-3}$ MOLS PER KG. PROTEIN | As/P x$10^4$ |
|---|---|---|---|---|
| **Chimpanzee #112** | | | | |
| Whole liver | 100 | 56 | 133 | .42 |
| Sol. in 1% NaCl | 82 | 60 | | |
| Sol. 1% NaCl; ins. $H_2O$ | 4 | 76 | 152 | .50 |
| Sol. $H_2O$; ins. .6 M* | 13 | 69 | 163 | .42 |
| Sol. .6 M; ins 1.2 M* | 15 | 61 | 226 | .27 |
| Sol. 1.2 M; ins. 1.8 M* | 19 | 43 | 61 | .70 |
| Sol. 1.8 M; ins. 2.7 M* | 17 | 28 | 62 | .45 |
| Sol. 2.7 M; ins. 3 6 M* | 2 | 23 | 61 | .38 |
| Sol. 3.6 M; ins. 10% $CCl_3COOH$† | 4 | 27 | 40 | .67 |
| **Baboon** | | | | |
| Whole liver | 100 | 30 | 123 | .24 |
| Nucleoprotein | .7 | 28 | 1350 | .02 |

* Molarity of ammonium sulfate.

† Precipitate with trichloracetic acid from the last supernatant removed from previous fraction.

tein fractions than in the more soluble fractions. Similar results were observed for other liver samples and a kidney specimen. Although a relationship seemed to exist between solubility and arsenic content, the proteins initially insoluble in 1% saline were not found to be any higher in arsenic than those fractions initially soluble in the dilute sodium chloride solution. The tendency for the values for arsenic and phosphorus to parallel each other in the liver (table 2) was not found when kidney tissue was investigated, for in that tissue the highest phosphorus values were obtained in the most soluble fractions.

In regard to nucleoprotein, the arsenic concentration did not appear to be any higher than the average obtained for other proteins (see Table 2).

TABLE 3

*Removal of arsenic and phosphorus from liver proteins under various conditions*

| TREATMENT | As PER CENT INITIAL VALUE | P PER CENT INITIAL VALUE |
|---|---|---|
| Chimpanzee #86 | | |
| Rapid isolation with 10% $CCl_3COOH$ | 100 | 100 |
| 10% $CCl_3COOH$ 18 hours 25°C | 105 | 32 |
| N/10 HCl ¼ hour 100°C | 48 | 70 |
| N/10 HCl 1 hour 100°C | 33 | 47 |
| 4% Borax 1 hour 25°C | 55 | 80 |
| N/10 NaOH 1 hour 25°C | 4 | 98 |
| Baboon | | |
| *Rapid isolation with 10% $CCl_3COOH$* | *100* | |
| pH 7.4 1 hour 25°C | 97 | 100 |
| pH 8.0 1 hour 25°C | 91 | |
| pH 8.5 1 hour 25°C | 85 | |
| pH 9.0 1 hour 25°C | 75 | 101 |
| pH 9.7 1 hour 25°C | 60 | |
| pH 12.5 1 hour 25°C | 35 | 87 |
| N/10 HCl 0.16 hour 100°C | 56 | 56 |
| N/10 HCl 0.5 hour 100°C | 41 | 47 |
| N/10 HCl 1.0 hour 100°C | 38 | 34 |
| N/10 HCl 2.0 hours 100°C | 35 | 34 |

Evidence against the replacement of phosphorus in proteins by arsenic may be found in the difference in the behavior of the two elements on hydrolysis (Table 3). In contrast to phosphorus, arsenic appears to be very susceptible to the action of cold alkali, and insensitive to cold acid, a behavior compatible with a combination with sulfhydryl groups. On the other hand, both arsenic and phosphorus show a similar behavior toward hot acid, for after a decrease to about one-third the original value a limit appears to be reached. This suggests the presence of two types of bonds for both elements in their combination with protein.

## SUMMARY

1. Following the administration of arsenite tagged with $As^{74}$, the distribution of arsenic in various fractions of mammalian tissues has been investigated.

2. The bulk of the arsenic was found in the protein fraction, with a much smaller amount in the acid-soluble portion and only an insignificant amount in the lipid fraction.
3. The arsenic was not evenly distributed throughout the proteins, but in general appeared to be more concentrated in those tissue proteins which are precipitated by less than half-saturated ammonium sulfate.
4. The nucleoproteins did not take up more arsenic than proteins in general.
5. From the comparative distribution and behavior on hydrolysis of phosphorus and arsenic, there is little evidence for the replacement of phosphorus by arsenic in the tissues.

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# THE COMPARATIVE THERAPEUTIC ACTIVITY OF SULFONAMIDES AGAINST BACTERIAL INFECTIONS IN MICE[1]

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In recent publications from this laboratory, we have reported on a method for the quantitative evaluation of the effectiveness of sulfonamides in streptococcus, pneumococcus, and colon bacillus infections in mice and on the comparative therapeutic activity of certain sulfonamides in these infections (1–5). The present investigation is a continuation of these studies and presents the comparative therapeutic activity of typical derivatives of sulfanilamide on streptococcus and pneumococcus infections in mice. *In vitro* data are included for comparison. By basing activity upon blood concentration of drug rather than on dosage *per os*, it was believed that more accurate data than have hitherto been available could be obtained on the relation of chemical constitution to *in vivo* activity and on the relation of *in vivo* to *in vitro* activity.

METHODS. The procedure for the collection of data and treatment of results was similar to that previously used (2–4). Whenever possible, Median Survival Doses (S.D.$_{50}$) and Median Survival Blood Concentrations (S.B.C.$_{50}$) were determined. Sulfanilamide was used as a standard of comparison in the streptococcus infection, sulfapyridine in the pneumococcus infection. When a compound was not sufficiently active to permit determination of an S.D.$_{50}$ an estimate of the activity ratio was obtained on the basis of survival times, using blood concentrations and not drug intakes *per os* for the comparison. Since comparisons in the present study are based on blood concentrations, inactivity of a drug cannot be explained by poor absorption from the intestinal tract.

CFCW mice were used in all of the experiments with the exception of experiment 13 (CFI mice). Three to five drug-diets and 40 to 50 mice were used for each drug in any particular experiment. In the complete study a total of about 3000 mice was used.

The C 203 strain of $\beta$-hemolytic streptococcus was used to produce the experimental streptococcus infection. The infection in experiment 12 consisted of about 20,000 lethal doses: in all other experiments, of about 200 lethal doses. The Neufeld strain of type I pneumococcus was used to produce the experimental pneumococcus infection. This infection in all experiments consisted of about 200 lethal doses.

Blood concentrations of drugs were determined as previously described (2). A calibration curve obtained with sulfanilamide was used in determining the different sulfonamide compounds. With every compound a factor for conversion of the sulfanilamide value to that of its derivatives was determined with three concentrations of standard solution. Usually this conversion factor agreed within an error of a few per cent with that calculated from the molecular weight of the compound. These experimentally determined factors were used in all calculations of drug concentrations in blood. When determining the factor the time necessary for maximum color development and the permanency of the color for 15 minutes was measured. In a few cases rapid fading of color occurred due to precipita-

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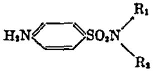
[2] Lalor Foundation Fellow.

tion of the azo dye; this could be eliminated by adding alcohol to the solution before coupling. Over 90 per cent recovery was obtained with all compounds used in this study.

The *in vitro* determinations of activity on streptococcus and *E. coli* were performed by the methods already described (5–7). The activity of each sulfonamide against the test strain of streptococcus (C 203) was measured in terms of the smallest concentration of drug required to inhibit the growth of an inoculum of about 200 bacteria per cubic centimeter in a peptone-dextrose broth at 39°C. for 48 hours. Against the test strain of *E. coli* (MacLeod), drug activity was measured in terms of the smallest concentration required to inhibit the growth of an inoculum of about 1000 bacteria per cubic centimeter in a synthetic medium at 37° for 48 hours. In each case, activity ratios have been based upon the minimal inhibitory drug concentrations (molar), using sulfanilamide as the reference standard. As previously reported (5) the *in vitro* activity ratios are probably accurate to within plus 50 and minus 25%.

TABLE 1

*$N^1$-Acyclic sulfanilamides*



| NO. | R₁ | R₂ | EXP. NO. | IN VIVO STREPTOCOCCUS | | | | | IN VITRO ACTIVITY RATIO | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $S.D._{50}$ | $S.B.C._{50}$ | Activity ratio *weight basis* | Activity ratio | | Streptococcus *molar basis* | E. coli *molar basis* |
| | | | | | | | *Molar basis* | *Range for limits of ±2 S.E.* | | |
| | | | | *mgm.* | *mgm. %* | | | | | |
| 76 | OH* | H | 1 | 5.2 | 1.6 | 1.1 | 1.1 | .6–2.3 | .02 | .06 |
| 182 | CH$_3$ | H | 2 | 20 | 5.6 | .46 | | | | |
| | | | 3 | 13 | 4.6 | .15 | .3 | .2– .5 | .3 | .5 |
| 191 | CH$_3$ | CH$_3$ | 4 | 44 | 5.8 | .21 | .3 | 1– .4 | | .6 |
| 46 | CH$_2$CH$_2$OH | H | 5 | 38 | 5.3 | .26 | .3 | .2– .6 | .2 | .2 |
| 74 | CH$_2$CH$_2$OH | CH$_2$CH$_2$OH | | | | † | | | | .2 |
| 172 | C=NH·NH$_2$ | H | 5 | 22 | 1.7 | .82 | | | 1 3 | 1.3 |
| | | | 6 | 13 | 1.8 | .56 | .8 | .5–1.3 | | |
| 183 | C=NH·NHCH$_3$ | H | 5 | 18 | 1 8 | .78 | 1.0 | .5–1.9 | .7 | 1.3 |
| 60 | COCH$_3$ | H | 7 | 34 | 2.5 | .44 | .6 | .3–1.0 | .3 | 5 |
| 106 | CO(CH$_2$)$_{10}$CH$_3$* | H | 1 | 13 | 2.6 | .69 | | | | |
| | | | 8 | 7.4 | .75 | .80 | | | | |
| | | | 9 | 1.6 | .98 | 1.6 | 1.0 | .6–1.7 | 4. | <.1 |
| 97 | CH$_2$COOH | H | | | | ‡ | | | 02 | .04 |

* Blood concentrations calculated as sulfanilamide.

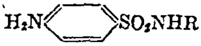
† Almost inactive.

‡ Less than $\frac{1}{10}$ as active as sulfanilamide.

RESULTS. The results of the present investigation are summarized in tables 1–8. In tables 1–5 are given the activity ratios of various sulfanilamide derivatives against the β-hemolytic streptococcus infection in mice as well as the *in vitro* activity against the same strain of streptococcus and the strain of *E. coli*. Sulfanilamide was used as the standard of comparison in these tests and its ac-

## TABLE 2

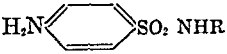
*$N^1$-Isocyclic sulfanilamides*

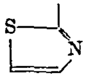
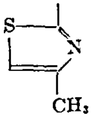
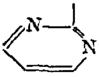
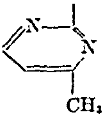
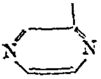


| NO. | R | IN VIVO ACTIVITY RATIOS: STREPTOCOCCUS | IN VITRO ACTIVITY RATIOS: Streptococcus, *molar basis* | E. coli, *molar basis* |
|---|---|---|---|---|
| 87 | $C_6H_5$ | About 0.05 | 3 | 6 |
| 167 | $C_6H_4OH$-2 | Less than 0.05 | .8 | 13 |
| 33 | $C_6H_4OH$-3 | Less than 0.05 | 3 | |
| 169 | $C_6H_4OH$-4 | Less than 0.05 | 1.5 | 1.5 |
| 105 | $C_6H_4NH_2$-2 | Less than 0.05 | .2 | 1.5 |
| 79 | $C_6H_4NH_2$-4 | Less than 0.05 | .8 | 3 |
| 6 | $C_6H_4COOH$-2 | Less than 0.05 | 3 | .2 |
| 8 | $C_6H_4COOH$-3 | Less than 0.05 | .4 | .4 |
| 4 | $C_6H_4COOH$-4 | Less than 0.05 | 3 | .03 |
| 5 | $C_6H_4SO_2OH$-2 | Inactive or almost so | .1 | .01 |
| 7 | $C_6H_4SO_2OH$-3 | Inactive or almost so | 1 | .02 |
| 9 | $C_6H_4SO_2OH$-4 | Inactive or almost so | .2 | .05 |
| 36 | $C_6H_4SO_2NH_2$-4 | See table 5 | | |

## TABLE 3

*$N^1$-Heterocyclic sulfanilamides*

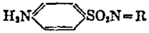


| NO. | R | EXP. NO. | IN VIVO STREPTOCOCCUS: $S.D._{50}$ | $S.B.C._{50}$ | Activity ratio *weight basis* | Activity ratio: *Molar basis* | *Range for limits of ± 2 S.E.* | IN VITRO ACTIVITY RATIO: Streptococcus *molar basis* | E. coli *molar basis* |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *mgm.%* | | | | | |
| 165 | <br> | 6 | 4.0 | 1.9 | .53 | .8 | .4–1.4 | 6 | 100 |
| 39 | <br> | 1 | 3.4 | 4.3 | .42 | .6 | .4–1.1 | | 50 |
| 168 | <br> | 10 | 1.3 | 2.5 | .44 | | | | |
| | | 11 | 1.2 | 1.9 | .84 | | | | |
| | | 12 | 3.0 | 4.7 | .79 | | | | |
| | | 13 | 2.1 | 2.4 | .62 | .9 | .6–1.3 | | 100 |
| 180 | <br> | 2 | 2.0 | 4.8 | .54 | | | | |
| | | 10 | .9 | 4.3 | .26 | | | | |
| | | 11 | 1.2 | 5.7 | .28 | | | | |
| | | 12 | 1.8 | 5.7 | .65 | | | | |
| | | 13 | 1.9 | 5.4 | .28 | .5 | .4– .7 | | 50 |
| 213 | <br> | 14 | .7 | 2.9 | .34 | .5 | .2–1.2 | | |
| 102 | Creatinine | | | | | | | | |

tivity was taken as unity. The values for the activity of sulfanilamide in the different experiments are given in table 6. In this table the "Experiment No.'s" correspond to those given in tables 1–5. The Median Survival Doses ($S.D._{50}$'s) are given in mgm. per mouse per day, and the Median Survival Blood Concentrations ($S.B.C._{50}$'s) in mgm. %, but the weighted activity ratios are expressed on a molar basis. In the arrangement of the tables Northey's (8) classification of sulfanilamide derivatives has been followed.

TABLE 4

*$N^1$-Heterocyclic sulfanilamides (continued)*



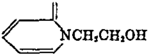
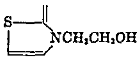
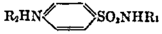
| NO | R | EXP. NO | IN VIVO STREPTOCOCCUS | | | | | IN VITRO ACTIVITY RATIO | |
|---|---|---|---|---|---|---|---|---|---|
| | | | S D 50 | S.B.C.50 | Activity ratio *weight basis* | Activity ratio | | Streptococcus *molar basis* | E coli *molar basis* |
| | | | | | | *Molar basis* | *Range for limits of ± 2 S.E.* | | |
| | | | *mgm.* | *mgm %* | | | | | |
| 121 |  | 5 | 51 | 2.8 | .50 | .8 | .4–1.5 | .4 | .2 |
| 192 |  | 4 | 11 | 1.6 | .75 | 1.4 | .8–2.5 | | |

TABLE 5

*$N^1$,$N^4$-Substituted sulfanilamides*



| NO. | $R_1$ | $R_2$ | EXP. NO. | IN VIVO STREPTOCOCCUS | | | | | IN VITRO ACTIVITY RATIO | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | S.D.50 | S.B.C.50 | Activity ratio *weight basis* | Activity ratio | | Streptococcus *molar basis* | E. coli *molar basis* |
| | | | | | | | *Molar basis* | *Range for limits of ± 2 S.E.* | | |
| | | | | *mgm.* | *mgm.%* | | | | | |
| 51* | H | $CH_2C_6H_5$ | 3 | 58 | 1.6 | .44 | .5 | .2–1.3 | | |
| 185* | OH | $CO(CH_2)_4CH_3$ | 7 | 15 | 1.9 | .58 | | | | |
| | | | 9 | 7.9 | 1.0 | 1.6 | 1.0 | .6–1.6 | | |
| 36 | $C_6H_4SO_2NH_2$-4 | H | 2 | 20 | 11 | .24 | | | | |
| | | | 3 | 6.0 | 1.4 | .50 | .7 | .4–1.2 | | |

* All blood concentrations calculated as sulfanilamide.

In the case of compounds Nos. 76 and 106 in table 1 and Nos. 51 and 185 in table 5, there is evidence that the arylamine present in the blood is sulfanilamide. No. 76, $N^1$-hydroxysulfanilamide, when diazotized and coupled takes about 10 minutes for the development of maximum color, whereas the arylamin found in blood after administering this drug resembles sulfanilamide in the rapidity of color development. No. 106, $N^1$-dodecanoylsulfanilamide, when

TABLE 6

*Activity of sulfanilamide in a streptococcus infection*

| EXP. NO. | $\beta$ ± S.E. | S.D.$_{50}$ | S.B.C.$_{50}$ | STANDARD ERRORS OF LOGARITHMS OF: | |
|---|---|---|---|---|---|
| | | | | S.D.$_{50}$ | S.B.C.$_{50}$ |
| | | *mgm.* | *mgm.%* | | |
| 1 | 2.7 ±1.0 | 3.4 | 1.8 | .082 | .102 |
| 2 | 2.9 ±1.2 | 6.2 | 2.6 | .079 | .116 |
| 3 | 1.6 ±1.0 | 2.7 | .7 | .128 | .133 |
| 4 | 5.0 ±1.3 | 3.8 | 1.2 | .051 | .079 |
| 5 | 2.4 ±1.6 | 4.0 | 1.4 | .109 | .114 |
| 6 | 2.5 ±0.9 | 3.2 | 1.0 | .089 | .095 |
| 7 | 4.0 ±1.1 | 2.8 | 1.1 | .078 | .098 |
| 8 | 1.3 ±1.6 | 2.5 | .6 | .141 | .147 |
| 9 | 1.9 ±1.2 | 4.7 | 1.6 | .106 | .113 |
| 10 | 2.1 ±1.0 | 3.3 | 1.1 | .097 | .102 |
| 11 | 1.6 ±2.0 | 4.5 | 1.6 | .129 | .135 |
| 12* | 1.5 ±0.7 | 11 | 3.7 | .133 | .143 |
| 13† | 4.1 ±1.6 | 7.4 | 1.5 | .057 | .094 |
| 14 | 2.2 ±1.2 | 3.6 | 1.0 | .116 | .142 |

* Infection = 20,000 lethal doses.

† CFl instead of CFCW mice.

TABLE 7

*Activity against pneumococcus*

| NO. | NAME | EXP. NO. | S.D.$_{50}$ | S.B.C.$_{50}$ | ACTIVITY RATIO WEIGHT BASIS | ACTIVITY RATIO | |
|---|---|---|---|---|---|---|---|
| | | | | | | *Molar basis* | *Range for limits of ± 2 S.E* |
| | | | *mgm.* | *mgm.%* | | | |
| 46 | $N^1$-($\beta$-hydroxyethyl) sulfanilamide | 1 | 100 | 18 | .40 | .5 | .4– .7 |
| 168 | Sulfadiazine | 2 | 8.6 | 10 | 1.1 | | |
| | Sulfadiazine | 3 | 15 | 20 | .65 | 1.2 | .8–2.0 |
| 180 | Sulfamethyldiazine | 2 | 9.2 | 13 | .85 | | |
| | Sulfamethyldiazine | 3 | 14 | 17 | .76 | 1.2 | .8–1.8 |
| 213 | Sulfapyrazine | 4 | 7.8 | 10 | 1.4 | | |
| | Sulfapyrazine | 5 | 5.9 | 8.8 | 1.7 | 2.3 | 1.5–3.4 |
| 2 | Sulfapyridine | 1 | 14 | 7.1 | | | |
| | Sulfapyridine | 2 | 20 | 11 | | | |
| | Sulfapyridine | 3 | 28 | 13 | | | |
| | Sulfapyridine | 4 | 31 | 14 | | | |
| | Sulfapyridine | 5 | 33 | 15 | | | |

given *per os* to a dog gives rise to sulfanilamide in the urine (see chemical data). No. 51, $N^4$-benzylsulfanilamide, can not be diazotized and coupled until decomposed to sulfanilamide. No. 185, $N^1$-hydroxy-$N^4$-hexanoylsulfanilamide, must be deacylated before it can be diazotized and the $N^1$-hydroysulfanilamide thus formed presumably is changed to sulfanilamide. The blood concentrations of these four compounds have been expressed as sulfanilamide.

In table 7, data are presented on the activity of four of the compounds against the pneumococcus infection in mice. In this case, sulfapyridine was used as the standard of comparison. The figures for the S.D.$_{50}$'s and S.B.C.$_{50}$'s of sulfapyridine in the five experiments are given at the end of the table.

The activity ratios of eight sulfonamide compounds on the streptococcus, pneumococcus and *E. coli* infections in mice are given in table 8. The activity ratios are based on the blood concentrations of the drugs necessary to produce the same therapeutic response, in other words on the S.B.C.$_{50}$'s. The ratios are given both in terms of weight and molar concentrations. The figures given in table 8 for some of the compounds have been calculated from data given in previous publications (2, 3, 5).

TABLE 8

*Activity on streptococcus, pneumococcus and E. coli infections*

| NO. | NAME | ACTIVITY RATIOS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Streptococcus | | Pneumococcus | | E. coli | |
| | | *Weight* | *Molar* | *Weight* | *Molar* | *Weight* | *Molar* |
| 1 | Sulfanilamide | 1 | 1 | 1 | 1 | 1 | 1 |
| 46 | $N^1$($\beta$-hydroxyethyl)-sulfanilamide | .3 | .3 | .9 | 1.1 | | |
| 172 | Sulfaguanidine | .8 | 1 | 2 8 | 3.5 | | |
| 2 | Sulfapyridine | 1 1 | 1.6 | 2.3 | 3.4 | 6 | 9 |
| 165 | Sulfathiazole | .6 | .9 | 2.8 | 4 2 | 10 | 15 |
| 168 | Sulfadiazine | 6 | 9 | 2 | 3 | 11 | 16 |
| 180 | Sulfamethyldiazine | .3 | .5 | 1.8 | 2.9 | | |
| *213* | *Sulfapyrazine* | .3 | .5 | *3.6* | *5.4* | | |

Discussion. Thirty-three typical sulfanilamide derivatives have been examined in the present investigation for their activity on a streptococcus infection of mice. The comparative therapeutic activity of the compounds has been based upon the blood concentrations which give the same therapeutic response.

On a weight basis (mgm. per cent in the blood) none of the derivatives are significantly more active than the parent compound, sulfanilamide. Several of the compounds which are shown in this study to be definitely less active have been reported by others as being equally or more active than sulfanilamide (8). However, to date, no sulfanilamide derivative has been shown to be more active in a streptococcus infection than the parent substance when satisfactory methods have been used for making the comparison. When compared on a molar basis, sulfapyridine (No. 2, table 8) and possibly *3*-$\beta$-hydroxyethyl)-*2*-sulfanilimido-

*2,3*-dihydrothiazole (No. 192, table 4) were more active than sulfanilamide. In the $N^1$-acylic derivatives (table 1) the only compounds which appear to be as active as sulfanilamide are the guanidine and methylguanidine derivatives (Nos. 172 and 183) and the two compounds which decompose to sulfanilamide in the body (Nos. 76 and 106). The $N^1$-isocyclic derivatives are all much less active than the parent compound with the exception of $N^1$-sulfanilylsulfanilamide (No. 36, tables 2 and 5). The $N^1$-heterocyclic derivatives (tables 3 and 4) [with the exception of No. 192 (table 4) and sulfapyridine (table 8)] are less active than sulfanilamide.

The data presented in table 8 indicate that there is a definite specificity in the action of sulfanilamide derivatives on bacterial infections in mice. While no derivative (with the exception of No. 2) appears to be more active than sulfanilamide on the streptococcus infection, all derivatives (with the exception of No. 46) are more active than the parent compound against the pneumococcus infection. Also, sulfapyridine, sulfathiazole and sulfadiazine are all much more active than sulfanilamide against the *E. coli* infection.

In a recent communication from this laboratory (9) it was shown that no compound which was inactive *in vitro* was active *per se* against a streptococcus infection *in vivo*, and a compound which was active *in vitro* might be either active or inactive *in vivo*. The conclusions of that qualitative study are confirmed by the present quantitative comparison of *in vivo* and *in vitro* activity. From the data as a whole, it is further evident that there is no quantitative relation between *in vivo* and *in vitro* activity on the streptococcus. While there is no correlation between *in vitro* activity upon the streptococcus and that upon the colon bacillus, no compound which was less active against coli *in vitro* was as active as sulfanilamide *in vivo* on the streptococcus with the exception of No. 121 (table 4).

Table 6 illustrates the variability in the response of the streptococcus infection to treatment with sulfanilamide and emphasizes the necessity for the use of some drug as a standard of comparison in every experiment. Omitting experiment 12, where a larger infecting dose was used, the Median Survival Blood Concentrations vary from 0.6 to 2.67 mgm. %; sulfanilamide is four times as active in one experiment as in another. In table 7, the variability of the response of the pneumococcus infection to sulfapyridine is seen to be about two-fold (S.B.C.$_{50}$ varies from 7.1 to 15 mgm. %).

CHEMICAL SECTION. Compounds no. 2, 4, 5, 6, 7, 8, 9, 106, 168, 172 and 180 were supplied by the American Cyanamid Company; nos. 1, 46 and 76 by Eli Lilly and Company; no. 213 by Mead Johnson and Company; no. 51 by Merck and Company, Inc., no. 60 by Schering Corporation; no. 185 by Sharp and Dohme; no. 165 by E. R. Squibb and Sons; and nos. 36 and 39 by Winthrop Chemical Company. We wish to thank the above for their courtesy in supplying these drugs. Compounds no. 33, 74, 79, 87, 97, 105, 167 and 169 were prepared in this laboratory. Compounds no. 182, 183, 191 and 193 are described below and compounds no. 121 and 192 will be described in a publication by Shepherd, Bratton and Blanchard (10).

*$N^1$-Methylsulfanilamide.* Eight-tenths of a mole of acetylsulfanilyl chloride was added in six portions to 0.8 mole of 33% aqueous methylamine in 450 cc. of 1 N $Na_2CO_3$ solution

maintained at 40–45°. The reaction mixture was kept at pH 8 to 9 by addition of 12 N NaOH. After disappearance of the solid, the $N^1$-methyl-$N^4$-acetyl-sulfanilamide was precipitated with 6 N HCl. Yield: 85–90%. M.p. 190.0–190.5° after recrystallization from water. Diazotization following de-acetylation gave 0.752 as much color as an equal weight of sulfanilamide; calculated *colorimetric factor:* 0.754.

Hydrolysis with 2 moles of 3 N NaOH for 1½ hours or with 2 moles of 2.5 N HCl for 15–20 minutes gave 75–85% yields of $N^1$-methylsulfanilamide. M.p. 112.5–113.0° (recryst. from water). *Colorimetric factor:* calc., 0.925; found, 0.927.

*$N^1$,$N^1$-Dimethylsulfanilamide* To 1 mole of dimethylamine hydrochloride, dissolved in a minimal amount of water, was added 3 moles of 12 N NaOH. An acetone solution of 1 mole of acetylsulfanilyl chloride was added slowly with stirring, keeping the temperature below 30°C. by addition of ice. The clear solution was neutralized and chilled, whereupon an oily upper layer separated and slowly crystallized. A portion of the product, $N^1$,$N^1$-dimethyl-$N^4$-acetyl-sulfanilamide, melted at 142–3° when recrystallized several times from water.

The acetyl compound was hydrolyzed by boiling ½ hour with 4 moles of 6 N HCl. The product, $N^1$,$N^1$-dimethylsulfanilamide, was recrystallized from 50% alcohol with the aid of charcoal and melted at 171–2°. The yield was 80 per cent, based on dimethylamine. *Colorimetric factor:* calc., 0.86; found, 0.86.

*$N^1$-(Methylguanyl)-sulfanilamide* Two hundred grams of Eastman's methylguanidine nitrate (1.47 moles) was dissolved in 313 cc. of 12 N NaOH, mixed with 580 cc. of acetone cooled to 10–15°. To this 350 grams of acetylsulfanilyl chloride (1.5 moles) in 1,000 cc. of acetone was added with stirring during 1 hour. After stirring 1 hour longer, the reaction precipitate (35–55 gm.) was separated by filtration This material was shown to be sodium $N^1$-methyl-$N^4$-acetylsulfanilamide by formation of $N^1$-methyl-$N^4$-acetyl-sulfanilamide by neutralization and of $N^1$-methylsulfanilamide by hydrolysis. The filtrate was neutralized and evaporated to dryness in a stream of air. Sufficient water was added to make the resultant syrup filterable and the product was obtained by suction-filtration, washing with dilute alkali and water. Yield. 12–16%. M.P. 246–7° (recryst. from 20% ethanol and dried at 125°). *Colorimetric factor* (after hydrolysis): calc., 0.637; found, 0.644.

Hydrolysis with 2½ moles of 1 N HCl for 15 minutes gave 50–65% yields of $N^1$-(methylguanyl)-sulfanilamide. M.p. 170.0–170.5° (recryst. from 20 per cent ethanol and dried at 125°). *Colorimetric factor:* calc., 0.754; found, 0.768. *Anal.* Calc. for $C_8H_{12}N_4O_2S$: C, 42.09; H, 5.30; found, C, 41.4; H, 5.24. The solubility in water at 37° is 510 mgm. %.

By alkaline cleavage to sulfanilamide the methyl group was shown to be on one of the terminal nitrogens of the guanidine group $N^1$-(methylguanyl)-sulfanilamide (0.01 mole) was refluxed 2 hours with 0.1 mole of 1 N NaOH. After neutralization and evaporation to dryness, the product was washed with cold water. The sulfanilamide was separated from unchanged starting material by treatment with dilute alkali and obtained in 65–75% yield by neutralization with 12 N HCl. M.p. 165–6° unchanged by mixing with authentic sulfanilamide. *Colorimetric factor:* calc., 1.00; found, 0.998.

*Sulfanilylcreatinine.* Twenty-three grams of creatinine (0.2 mole) was dissolved in 100 cc. of water at 40–50°. To this 46.6 grams of acetylsulfanilyl chloride (0.2 mole) was added in small portions, using 12 N NaOH to keep the mixture at pH 8–9. Neutralization with 12 N HCl gave 8–12 grams of product (13–19% yield). M.p. 266° (dec.) after recrystallization from water. *Colorimetric factor* (after hydrolysis): calc., 0.555; found, 0.55.

Hydrolysis with 3.5 moles of 2 N HCl for 35 minutes gave 80–85% yields of sulfanilylcreatinine. M.p 228° (dec.) after recryst. from water. *Colorimetric factor:* calc., 0.642; found, 0.672. *Anal* Calc. for $C_{10}H_{12}N_4O_3S$: C, 44.77; H, 4.51; found, C, 44.90; H, 4.49.

*Hydrolysis of $N^1$-Dodecanoylsulfanilamide to Sulfanilamide in the Dog.* A 20 kgm. dog was given by stomach tube 4 grams (0.2 gm./kgm.) of $N^1$-dodecanoylsulfanilamide suspended in olive oil. The urine, collected during the following 23 hours, amounted to 300 cc. and contained 450 mgm. of arylamine, expressed as sulfanilamide. The urine was treated with 2 grams of Norite and allowed to stand 18 hours in the refrigerator. The charcoal was filtered off, and analysis of the filtrate indicated 300 mgm. of arylamine (expressed as sulfanilamide) had been adsorbed on the charcoal. The charcoal was extracted 6 hours in a Soxhlet extractor with alcohol, and the 54 cc. of extract contained 280 mgm. of arylamine (expressed as sulfanilamide). After evaporation to a syrup, the residue was taken up in 10 cc. of hot water containing a little $NaHCO_3$. Chilling yielded 172 mgm. of a product melting at 164–5°. After recrystallization from water, it melted at 165–6°, unchanged by admixture with authentic sulfanilamide. Colorimetric analysis of the recovered sulfanilamide indicated a purity of 98%.

## SUMMARY

The therapeutic activity of 33 typical derivatives of sulfanilamide against a streptococcus infection in mice has been compared. With the exception of sulfapyridine none of these derivatives was found to be significantly more active than sulfanilamide, while most of them were less active than the parent compound. On a pneumococcus infection, sulfapyridine, sulfathiazole, sulfadiazine, sulfamethyldiazine, sulfapyrazine and sulfaguanidine are all more active than sulfanilamide. There appears to be a definite specificity in the action of sulfanilamide derivatives on bacterial infections in mice. Although all compounds which were active *in vivo* were also active *in vitro*, there appears to be no quantitative relation between *in vitro* and *in vivo* activity.

We wish to thank Dorothea Babbitt and Dorothy J. MacKenzie for technical assistance in this work.

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# STUDIES ON THE FATE OF NICOTINE IN THE BODY

## I. THE EFFECT OF pH ON THE URINARY EXCRETION OF NICOTINE BY TOBACCO SMOKERS

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Within recent years increased attention has been paid to the urinary elimination of nicotine in man, such studies having received particular impetus through the development of suitable qualitative and quantitative chemical methods of analysis. Fretwurst and Hertz in 1932 (1) were apparently the first to crystallize nicotine, as the dipicrate, from urine, and to determine the amount present quantitatively. Their observations were made in a gardener who had been severely poisoned by exposure to a nicotine insecticide spray. In 1933 Dingemanse and Freud (2) indicated that the substance "Katatonin," which they had isolated from human urine as a picrate and picrolonate, was identical with nicotine chemically and pharmacologically, and stated that their method of analysis for katatonin was equally suitable for nicotine. Bodnár, Nagy and Dickmann (3) reported in 1935 on the quantitative estimation of nicotine in the urine of smokers, and Perez (4) published similar observations several years later. In this country Helmer, Kohlstaedt and Page (5) prepared crystalline nicotine picrate from the urine of smokers in 1939, and subsequently Corcoran, Helmer and Page (6) developed two methods with which they studied quantitatively the amount of nicotine in the urine.

From the foregoing it is clear that some nicotine is excreted unchanged in the urine, but available evidence indicates that this is only a small proportion of the total amount absorbed. Preliminary to investigations concerned with the fate of the remainder, we have had occasion to study the urinary elimination of nicotine by tobacco smokers. We found striking and frequently inexplicable differences in the quantity of nicotine eliminated in the urine of smokers, and these pointed to the need for seeking the factors which might govern this excretion. Travell's (7) observations on the effect of different hydrogen ion concentrations on the absorption of nicotine from the urinary bladder of cats suggested that the factor of nicotine reabsorption from the urinary tract might be of major importance. Hence in this study we have particularly investigated the effect of acidification and alkalinization of the urine on the elimination of nicotine by tobacco smokers. All of the subjects studied were healthy, young adult males.

PROCEDURES. A. *Determination of nicotine in urine. Chemical:* The generally accepted method for the macro-determination of nicotine is the silicotungstic acid method of the A O A C. (8), and Spies (9) has shown that a method similar in principle is applicable to micro-quantities. The method we used was based on both of these technics, the principal adaptative modification consisting of concentrating the nicotine in proportion to the other urinary constituents by ether extraction from an alkaline urine.

Experiments on the recovery of small amounts (2, 5 and 10 mgm.) of nicotine added to 24 hour specimens of urine from smokers as well as from non-smokers, indicate that the error of the method is less than 10%, with a tendency towards high rather than low values.

Traces of a substance forming an insoluble silicotungstate by this method of analysis were found in the urine of two non-smokers (0.01 and 0.08 mgm. per 24 hour urinary specimen). These quantities were insufficient for identification. They may represent a slight blank on the determination, or nicotine absorbed from the atmosphere in the presence of smokers.

*Biologic:* A solution was made by regenerating the base from the insoluble silicotungstate obtained in the chemical analysis, extracting with ether and treating with dilute acetic acid. When injected into the ventral lymph sac of frogs (*Rana pipiens*) in amounts of 0.015 mgm. per 40 gram frog, it elicited the characteristic response of the limbs as described by Fühner (10). A control solution of nicotine acetate gave the same threshold of response. The material recovered from urine, when injected intravenously into an anesthetized dog, elicited a blood pressure response that was quantitatively and qualitatively identical with that produced by the control nicotine solution. For instance, the average blood pressure rise produced by 0.01 mgm. per kgm. of control nicotine was 70.6 mm. Hg, while with the same amount of the base the rise was 69.0 mm. Hg.

B. *Methods of acidifying and alkalinizing the urine.* For the purpose of maintaining the urine acid 1 gram of ammonium chloride was administered orally 4 times daily and 24 hour specimens of urine collected when the pH of the urine had fallen to or below 5.8 (glass electrode), at which point, according to Travell (7), there was no evidence of nicotine absorption from the urinary bladder of cats. To alkalinize the urine 5 grams of an effervescent alkaline citrate and carbonate preparation was given 4 times daily and specimens collected while the urine was maintained alkaline.

TABLE 1

*Daily urinary elimination of nicotine by smokers*

(pH of urine uncontrolled)

| | SUBJECT 1 | SUBJECT 1* | SUBJECT 2 | SUBJECT 3 | SUBJECT 4 |
|---|---|---|---|---|---|
| Cigarettes smoked daily (*about*) | 40 | 40 | 40 | 40 | 40 |
| Nicotine in urine (*mgm.*) | 2.2 | 7.5 | 2.2 | 7.6 | 4.8 |

* Average of four 24 hour periods.

RESULTS. Table 1 shows the amount of nicotine eliminated in the urine of smokers during the periods when no attempt was made to control the pH of their urine. The subjects were inhalers, but otherwise unselected as to smoking habits except for the fact that they all smoked approximately the same number of cigarettes per day.

It will be noted that the daily elimination of nicotine differed widely between the four subjects (2.2 to 7.6 mgm.), and even in the same subject (#1) on different occasions. In this latter smoker the values ranged from 2.2 mgm. for one 24 hour period to 7.5 mgm. as an average 24 hour value calculated from the results obtained on a pooled 96 hour urine collection.

In table 2 are shown the effects of acidifying and alkalinizing the urine on the elimination of nicotine by three smokers. It is immediately apparent that the urine, when alkaline, contained definitely less nicotine than when acid. In subject #1, for instance, the average daily elimination of nicotine was 2.65 mgm. when the urine was alkaline, 15 mgm. when the urine was acid. It is also to be observed that when acid the nicotine content of the urine was more nearly proportional to the number of cigarettes smoked. Had it been possible better to standardize the smoking habits of these subjects (i.e. completeness of smoking each cigarette, frequency of puffs, etc.) greater proportionality might have been found.

TABLE 2

*The effect of hydrogen ion concentration of urine on the amount of nicotine eliminated by tobacco smokers*

| SUBJECT | CIGARETTES SMOKED DAILY | AMOUNT NICOTINE RETAINED FROM SMOKE (CALCULATED) | pH OF URINE | NICOTINE ELIMINATED | PER CENT URINARY ELIMINATION OF NICOTINE RETAINED FROM SMOKE |
|---|---|---|---|---|---|
| | | *mgm* | | *mgm /24 hr.* | |
| 1<br>(inhaler) | 40 | 115 | 5.5 | 12.7 | |
| | | | 5.0 | 17.3 | |
| | | | | av. 15.0 | 13.04 |
| | | | 7.1 | 2.1 | |
| | | | 7.6 | 3.2 | |
| | | | | av. 2.65 | 2.30 |
| 7<br>(inhaler) | 18 | 52 | 5.6 | 5.37 | |
| | | | 5.7 | 6.27 | |
| | | | | av. 5.82 | 11.19 |
| | | | 7.1 | 2.09 | |
| | | | 7.2 | 1.30 | |
| | | | | av. 1.69 | 3.25 |
| 8<br>(inhaler) | 20 | 58 | 5.3 | 5.68 | 9.79 |
| | | | 7.3 | 2.17 | 3.74 |
| 9<br>(non-inhaler) | 40 | 14 | 4.9 | 0.24 | |
| | | | 5.2 | 3.00 | |
| | | | | av. 1.62 | 11.57 |

Table 2 also shows the results of studies made on a non-inhaler when the urine was maintained acid to minimize nicotine reabsorption. This non-inhaler eliminated only about 10 per cent as much nicotine as did subject #1 when both were under the same experimental conditions as to urinary pH and number of cigarettes smoked.

It may be pointed out that the daily volume of urine, although varying several fold between individuals, remained relatively constant for each subject during the several experimental periods.

Discussion. In order to calculate the percentage of nicotine eliminated in the urine of our subjects it was necessary for us to make certain assumptions as to the extent of nicotine retention during smoking. The main stream smoke from each of the cigarettes we used contained approximately 3 mgm. of nicotine, as determined by the method described by Bradford, Harlow, Harlan and Hanmer (11). On deep inhalation, Hillsman (12) found that from 90 to 98% of this nicotine is absorbed. Values of from 88 to 97% have been reported by others (3) (13) (14). An arbitrary value for the nicotine absorbed on inhalation of 2.88 mgm. (96% of 3 mgm.) per cigarette, is accepted here for calculations of the percentage eliminated in the urine of our subjects. In view of Hillsman's (12) observation that a non-inhaler retains, as an average, about 12% of the nicotine in the main stream smoke, we have assumed that the non-inhaler we studied absorbed 0.36 mgm. nicotine per cigarette.

Employing the above assumptions we found that about 2.5 to 4% of the retained nicotine was excreted in the urine when the urine was maintained alkaline, from 2 to 7% when the reaction of the urine was not controlled, and from 10 to 13% when it was maintained distinctly acid. The figures of Bodnár, Nagy and Dickmann (3) and Corcoran, Helmer and Page[1] (6) obtained on smokers in whom no attempt was made to control the urinary pH, fall approximately within the total range of these values. The finding of Bodnár, Nagy and Dickmann (3), that no nicotine appeared in the urine of a non-inhaler, was not confirmed by our observations on a non-inhaling subject in whom nicotine reabsorption from the urinary tract was inhibited by urinary acidification.

In regard to the relationship between the amount of nicotine retained during smoking and the quantity of nicotine excreted in the urine, correlation of a high degree could be expected only if the human organism remained in the same physiologic state. This is not usually the case, and from our data it appears that one of the important variants in this connection is that of urinary pH.

Since we found in the urine at the most only 15% of the nicotine retained from tobacco smoke, our results are in keeping with the current belief that some more potent mechanism than renal excretion must normally be active to care for the disposal of nicotine. The conclusion of Perez (4) that approximately all of the nicotine received by the smoker is excreted in the urine is vitiated by the dubious method used by him to determine the nicotine content of tobacco smoke.

Travell, Bodansky and Gold (16) have suggested the possibility of reabsorption of nicotine from the bladder of smokers, even to the point of poisoning if the urine became alkaline. We believe we have demonstrated reabsorption of nicotine from the urine of smokers when the urine was maintained alkaline. At what point along the urinary tract this occurs to the greatest extent is not known. However, it would seem that there is ordinarily little danger of poisoning from this source. Even when the urine of smokers is acid in reaction, thus minimizing reabsorption, a considerable volume would have to be present in the bladder to contain sufficient nicotine to be a potential source of poisoning. That such a volume could be shifted from an acid to an alkaline reaction suddenly enough to be effective, other than by acute experimental procedures, seems remote.

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[1] Their quotation of Wenusch (15) as having found 30 mgm. of nicotine in the smoke from one cigarette is an obvious typographical error; it should read 3.0 mgm.

As pointed out by Crane (17) in 1921, the effect of pH is exerted not so much on the absorbing tissue as on the drug, by determining the proportion of the drug in the free and combined states. Free nicotine is readily absorbed, while its ion is poorly absorbed, if at all. The ionization constant of nicotine is such that large changes in the ratio of free to combined base are produced by variations in hydrogen ion concentration within the range compatible with living tissues. This has been mentioned by Ellisor and Richardson (18), Travell (7), and by Weatherby (19).

In view of our observations it might be emphasized as a generality that, in studies of the extent of urinary excretion of a chemical, the reaction of the urine and the dissociation constant and relative resorbability of the free and combined drug, plus the possible effect of pH on the absorbing membrane, should be taken into considerations as possible influencing factors.

## SUMMARY

1. Nicotine has been determined quantitatively in human urine by a modified silicotungstic acid method.
2. During the periods when the urine was alkaline, the amount of nicotine excreted by tobacco smokers was only 25% of that noted when the urine was acid, presumably because of reabsorption of the nicotine from the urinary tract.
3. Data are presented supporting the view that some potent mechanism other than urinary excretion is active in the disposal of nicotine by tobacco smokers.
4. In studies of the quantitative urinary elimination of chemicals generally the possible influence of urinary pH on their resorption from the urinary tract might well be borne in mind.

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# STUDIES ON THE FATE OF NICOTINE IN THE BODY

## II. ON THE FATE OF NICOTINE IN THE DOG

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Up to 15% of administered nicotine is excreted unchanged in the urine (1, 2, 3, 4). The remainder is apparently detoxified in the animal organism. It is with the latter fraction that we are here concerned.

The important rôle of the liver in this detoxication process has been shown by Biebl, Essex and Mann (5) using the heart-lung-liver preparation. Although Wenusch (6) was unable to demonstrate detoxication of nicotine by finely hashed pig liver, Werle (7), testing the ability of isolated tissues of the rabbit (Warburg technique) to detoxify nicotine, found that liver is outstandingly active. Kidney and lung tissues were also found able to detoxify nicotine, whereas muscle, spleen, brain and small intestine mucous membrane showed no activity in this respect. Detoxication was dependent upon the presence of oxygen and did not proceed in an atmosphere of nitrogen or in the presence of 0.001–0.003 M of KCN or in oxygen containing 20% carbon monoxide. Werle concluded that detoxication of nicotine is dependent upon enzymatic processes.

The identity of the end product or products of nicotine detoxication in the animal organism is unknown. A single possibility has been suggested. Linneweh and Reinwein (8) in 1932 postulated nicotine as a possible precursor of the trigonelline of human urine. Apparent support for this appeared recently. Melnick, Robinson and Field (9), investigating the urinary excretion of nicotinic acid and related compounds as an index of the nutritional level of this dietary essential, observed a marked increase after smoking in the excretion of trigonelline by individuals on a basal diet. Their analytical procedure depends on the hydrolysis of trigonelline to a substance that forms a colored compound in the presence of cyanogen bromide and aniline. This color reaction is by no means specific, and is sensitive in varying degree to pyridine and many pyridine compounds. It is quite possible that the procedure used for the hydrolysis of trigonelline resulted in the conversion of the end product of nicotine detoxication to a substance yielding increased color with these reagents. This interpretation appears probable in view of the findings of Perlzweig, Levy and Starett (10) who used a somewhat different procedure for the hydrolysis of trigonelline and substituted metol for aniline in the color reaction. These authors, whose subjects also were maintained on a basal diet, found no difference in the quantity of trigonelline excreted by smokers and non-smokers.

It is generally accepted (11, 12, 13, 14) that administered pyridine is not further broken down in the body but is detoxified by methylation and appears in the urine as a salt of *1*-methyl pyridinium hydroxide. Thus alteration of the pyrrolidine component of the nicotine molecule would appear to be the most likely means of detoxication. Of the large number of possible nicotine derivatives involving various stages of change of the pyrrolidine component, relatively few are susceptible of determination short of isolation and subsequent identification. To date, known analytical procedures for such substances in impure solution are limited to those in which the pyrrolidine component has been completely removed (pyridine) or reduced to a one carbon side chain (nicotinic acid class).

As a starting point, therefore, we have administered nicotine to animals and examined their urine for pyridine derivatives involving maximal or near maximal degradation of the pyrrolidine component of nicotine, namely, *1*-methly pyridinium hydroxide and nicotinic acid and its metabolic end products, trigonelline and nicotinuric acid.

Experimental Procedure. Female dogs maintained on a constant daily ration of Purina Dog Chow were used in these experiments. Urine was collected at 24 hour intervals by catheterization. Following the collection of 3 consecutive 24 hour control urines, 15 hourly subcutaneous injections of a 2% solution of nicotine in the amount of 0 2 mg. per kg. each were given to each animal, the final dose coming 10 hours prior to catheterization. Two 24 hour collections of urine were made subsequent to the nicotine administration period.

An aliquot of the urine was analyzed for nicotine by a silicotungstic acid method (4). Nicotinic acid, nicotinuric acid and trigonelline were determined by the method of Perlzweig, Levy and Starett (10) and trigonelline and *1*-methyl pyridinium hydroxide by a modification[1] of the procedure of Kodicek and Wang (15). All color intensities were measured spectrophotometrically.

Results and discussions. The average results from experiments on four dogs are shown in figure 1. Of the average total of 28.35 mgm. of nicotine received by each dog only 2.65 mgm. was excreted unchanged in the urine. The remaining 25.7 mgm. is equivalent to 21.7 mgm. of trigonelline, or 17 mgm. of *1*-methyl pyridinium hydroxide. Since there was no increase in the excretion of trigonelline, this substance is obviously not an end product of nicotine detoxication in the dog.

The method of Kodicek and Wang, in our hands, is about one-fourth as sensitive to *1*-methyl pyridinium hydroxide as it is to trigonelline. Accordingly, had *1*-methyl pyridinium hydroxide been present in the urine following administration of nicotine, the apparent trigonelline excretion by the Kodicek method would have been considerably greater than that as determined by the Perlzweig procedure. Since this was not the case, *1*-methyl pyridinium hydroxide is apparently not an end product of nicotine detoxication.

The nicotinic acid fraction (N.A.F.) as determined on unhydrolyzed urine by the Perlzweig method, expressed in terms of molecular equivalents of nicotinic acid, includes all free nicotinic acid, 50% of any nicotinamide present, 20% of any nicotinuric acid, 10% of any nicotine, and unknown percentages of other pyridine derivatives, should they be present. By acid hydrolysis, however, nicotinamide and nicotinuric acid are converted to nicotinic acid, thereby increasing their contribution to the total color developed. Other unknown pyridine derivatives which might be present may or may not have their reactivity altered by this procedure. As a result of such alteration of chemical structure the N.A.F. acid hydrolyzed is greater in quantity than the N.A.F. unhydrolyzed.

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[1] This consisted primarily of substitution of a lead decolorization of the urine, similar to that used by Perlzweig, for the charcoal suggested by Kodicek, since all samples of charcoal tried by us adsorbed trigonelline.

As seen from figure 1, the N.A.F. acid hydrolyzed, on the administration of nicotine, increased from a control level of about 0.6 mgm. per day to 3.3 mgm. This increase is not thought to be due to an increase in the quantity of nicotinic acid since trigonelline, the chief end product of nicotinic acid metabolism, is unaffected. Nor is it thought to be due to nicotinuric acid or nicotinamide, since a corresponding increase occurred in the N.A.F. without hydrolysis (figure 1). Likewise the quantity of nicotine excreted unchanged (2.65 mgm.) at a sensitivity of 10% could contribute only about 0.26 mgm. to the observed increase. Apparently then, although a substance which we have tentatively assumed to be the end product of nicotine detoxication in the dog appears in the N.A.F. as measured by the Perlzweig method, it is not identical with any of the known substances that we set out to measure.

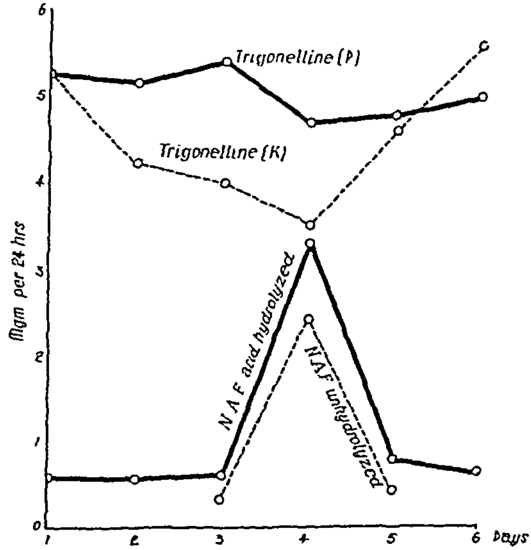

FIG. 1. INFLUENCE OF NICOTINE ADMINISTRATION ON THE EXCRETION OF CERTAIN PYRIDINE DERIVATIVES BY THE DOG

Average of results on 4 dogs. An average total of 28.35 mgm. of nicotine was administered to each dog on day 4. (*P*)—method of Perlzweig, Levy, and Starett. (*K*)—method of Kodicek and Wang. *N.A.F.*—nicotinic acid fraction.

In a repeat experiment in which the urine of all four dogs was pooled for analysis, 124.2 mgm. of nicotine was injected and 14.3 mgm. appeared unchanged in the urine. The N.A.F. acid hydrolyzed increased from a control level of about 4.0 mgm. per day to 15.4 mgm. The N.A.F. unhydrolyzed showed a corresponding increase while the trigonelline excretion was not significantly changed, confirming our previous results.

Two additional observations have been made that give considerable specificity to this unknown substance appearing in the urine of the dog after nicotine administration, and permit its presence to be distinguished from that of other pyridine derivatives normally found in dog urine. With cyanogen bromide and metol nicotinic acid gives a yellowish color which exhibits a minimum transmittance at a wave length of 400 mμ. After acid hydrolysis, the urine of the dog following nicotine administration exhibited similar color changes. However, when acid hydrolysis was omitted, a rose color, with a minimum transmittance at 495 mμ (figure 2), developed on the addition of cyanogen bromide alone. The addition of metol definitely altered this to a hue which absorbed at the wave length of the nicotinic acid color. Nicotine itself, with cyanogen bromide and without metol, yields a greenish yellowish color. We believe this rose color developed by cyanogen bromide to be characteristic of the detoxication product of nicotine in the dog, since in our experience it has never been observed in the urine of dogs not receiving nicotine.

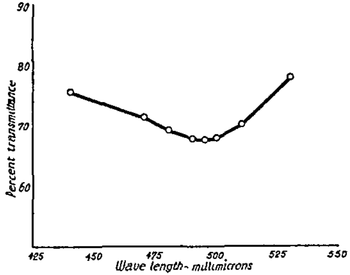

FIG. 2. ABSORPTION CURVE OF COLOR PRODUCED BY ADDITION OF CNBr TO UNHYDROLYZED URINE OF THE DOG FOLLOWING NICOTINE ADMINISTRATION

The second observation that serves further to distinguish this substance is that simple boiling of neutral urine containing it destroys its ability to yield a rose color on addition of cyanogen bromide.

## CONCLUSIONS

1. About 10% of nicotine given by subcutaneous injection to the dog is excreted unchanged in the urine.
2. The remainder appears, at least in part, in the nicotinic acid fraction of the urine as determined by the method of Perlzweig, Levy and Starett.

3. The detoxified nicotine is not converted to *1*-methyl pyridinium hydroxide, nicotinic acid, nicotinuric acid or trigonelline in measurable quantities.

4. After nicotine administration, the urine of the dog yields a rose color with cyanogen bromide. This property is lost if the urine has previously been boiled.

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# SOME TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES ON *3-5*-DINITRO-*o*-CRESOL

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In the search for effective substitutes for lead and arsenic for the control of insect pests on fruit trees and vegetables, attention is being constantly directed to the use of organic compounds which are more toxic for plant insects and presumably less toxic for plants, domestic animals, and man. During the past several years considerable interest has been revived in the use of nitrophenols, particularly with *3-5*-dinitro-*o*-cresol (apparently also known as *4-6*-dinitro-*o*-cresol). Although considerable studies have been reported on the insecticidal activity of this compound (1), (2), (3), (4), (5), comparatively no information is directly available dealing with its toxicity for mammals, other than studies on its action as a metabolic stimulant (6), (7), (8).

In view of the promising results obtained with *3-5*-dinitro-*o*-cresol as an insecticide, the determination of the possible health hazards to domestic animals and humans coming in contact with it, either accidentally or intentionally, is especially important. Accordingly, studies have been made on the acute, subacute, and chronic toxicity of *3-5*-dinitro-*o*-cresol after various routes of administration.

For this purpose studies were made on a pure sample of *3-5*-dinitro-*o*-cresol, sodium salt, which was obtained from Eastman Kodak Company.

EXPERIMENTAL PROCEDURES AND RESULTS. *Toxicity after subcutaneous injection.* While handlers of dinitro-*o*-cresol will not be exposed to the drug by this mode of administration, there remains the remote possibility that handlers with open sores may be exposed to it either in the form of a spray or in more concentrated form. Accordingly the acute toxicity after subcutaneous administration was determined in 152 rats of different sex and weighing 100 to 125 grams each. The levels injected and the data obtained are summarized in table 1.

In the rats that were injected with doses of 10 to 20 mgm. per kilogram no toxic symptoms were observed. With doses of 25 mgm. per kilogram all the rats showed a moderate degree of hyperactivity followed by depression and pyrexia. The average elevation in rectal temperature ½, 1, and 2 hours after injection was 2, 4, and 3°C. respectively. By the end of the fifth hour the temperature had returned to normal. All but four of the rats in this group survived and appeared perfectly normal 24 hours later. Rats receiving 30 to 50 mgm. per kilogram all became depressed, dyspneic, and showed signs of asphyxial convulsions. The majority became comatose and died, with *rigor mortis* setting in almost immediately following death. In rats receiving 50 mgm. per kilogram, in addition to the above symptoms cyanosis and marked edema of the tongue were invariably present. The minimum fatal dose (M.F.D.) which killed any animal was found to be 20 mgm. per kilogram.

The toxic effects after repeated subcutaneous injections were studied in 20 rats weighing approximately 100 grams each at the start. The rats were injected daily for a period of 30 days. Urine was examined at frequent intervals for sugar, albumin, and red cells. Hemoglobin and red blood cell count were determined at the beginning of the experiment and again on the day following the last injection. Urinary findings were negative, and the blood picture of the experimental animals was not significantly different from the controls. None of these animals showed any toxic signs during the experiment, but showed a slight increase in body weight as compared with controls. The average increase in weight for the injected rats was 46% as compared to 42% for the controls. All the injected rats were autopsied and examined grossly and microscopically. No gross changes were found at the site of injection or in the viscera.

For microscopic study the tissues were fixed in alcohol-formalin, embedded in paraffin and stained with hematoxylin-eosin. The following tissues were examined: urinary bladder, stomach, small intestine, kidney, liver, spleen, heart, and lung. On histological examination no significant pathological changes were found. It appears, therefore, that no chronic systemic toxicity or cummulative action is associated with repeated sublethal doses.

TABLE 1

*Minimal fatal dose for rats given subcutaneous injections of 3-5-dinitro-o-cresol, sodium salt*

| NUMBER OF ANIMALS | DOSE | DEAD | TIME UNTIL DEATH |
|---|---|---|---|
| | *mgm./kgm.* | *per cent* | *hours* |
| 20 | 10 | 0 | |
| 22 | 15 | 0 | |
| 20 | 20 | 5 | 12 |
| 30 | 25 | 13 | 2½ to 12 |
| 26 | 30 | 69 | 2 to 2½ |
| 15 | 40 | 100 | ½ to 1½ |
| 19 | 50 | 100 | ¼ to ½ |

*Toxicity after oral administration.* Sixty-nine male rats each weighing approximately 150 grams were given single doses of dinitrocresol ranging from 20 to 100 mgm. per kilogram by stomach tube. In rats receiving 20 mgm. per kilogram no toxic signs were observed. Rats receiving 30 mgm. per kilogram showed signs of depression, while those receiving the larger doses, 40, 50, and 100 mgm. per kilogram, showed varying degrees of depression. All the rats receiving the larger doses developed marked dyspnea, cyanosis, and asphyxial convulsions shortly before death, similar to those observed after subcutaneous administration of fatal doses. In table 2 are recorded the results of these observations. The M.F.D. was found to be 30 mgm. per kilogram.

The toxic effects of continued oral administration of dinitrocresol was studied in 75 young male albino rats of Wistar strain, of approximately the same age and weight. Two sets of feeding experiments were carried out. In the first series, which was started during the winter months, 9 groups of 5 rats each were used. One group served as control, and was fed the basic diet which had the following percentage composition: yellow corn meal 72, casein 10, linseed oil cake meal 10, ground alfalfa 2, bone ash 1.5, sodium chloride 0.5, Brewers' yeast 1, and cod liver oil (U.S.P.) 3. The remaining groups were given the same diet to which was added 0.00078, 0.00156, 0.00312, 0.00625, 0.0125, 0.025, 0.05, and 0.1% *3-5*-dinitro-*o*-cresol. In the second series, which was started in the spring, 6 groups of 5 rats each were used. One group served as control and the other groups were placed on diets containing 0.00312, 0.00625, 0.0125, 0.025, and 0.05% dinitrocresol. In all cases the rats were allowed free access to food and water at all times. Food consumption and rats were weighed once each week, at which time careful observations were made as to the condition of the animals. Rats surviving on these diets after 105 days were autopsied and examined grossly and microscopically.

TABLE 2

*Minimal fatal dose for rats given oral administration of 3-5-dinitro-o-cresol, sodium salt*

| NUMBER OF ANIMALS | DOSE | DEAD | TIME UNTIL DEATH |
|---|---|---|---|
| | *mgm./kgm* | *per cent* | *hours* |
| 10 | 20 | 0 | |
| 30 | 30 | 10 | 4 to 12 |
| 15 | 40 | 100 | 1 to 8 |
| 10 | 50 | 100 | ½ to 4 |
| 10 | 100 | 100 | 2 to 2½ |

TABLE 3

*Effect of feeding 3-5-dinitro-o-cresol to growing rats (105 days)*

| CONCENTRATION OF DINITRO-CRESOL IN DIET | NUMBER OF RATS | | AVERAGE WEIGHT | | AVERAGE (DAILY) FOOD CONSUMPTION | | AVERAGE (DAILY) DRUG CONSUMPTION |
|---|---|---|---|---|---|---|---|
| | At start | At end | At start | At end | *Per rat* | *Per kgm.* | |
| *per cent* | | | *grams* | *grams* | *grams* | *grams* | *mgm./rat* |
| Control | 10 | 10 | 55 | 319 | 12.5 | 58.7 | 0 |
| 0.00078 | 5 | 5 | 54 | 313 | 12.4 | 57.2 | 0.096 |
| 0.00156 | 5 | 5 | 55 | 300 | 12.3 | 58.5 | 0 19 |
| 0.00312 | 10 | 8 | 53 | 308 | 12.5 | 57.8 | 0.39 |
| 0 00625 | 10 | 8 | 54 | 335 | 14.4 | 64.2 | 0.90 |
| 0.0125 | 10 | 4 | 53 | 330 | 15 2 | 67.0 | 1.90 |
| 0 025 | 10 | 4 | 54 | 270 | 14.8 | 80.0 | 3.70 |

The results on growth and food consumption on the two series of rats, started at different times of the year, were essentially the same so that they may be considered together.

In table 3 data on rat weights, food consumption, and drug consumption are summarized. The growth of rats receiving 0 00078 to 0.00312% dinitrocresol in the diet closely parallels the growth of the control rats. Food consumption in these various groups was not significantly different. In rats receiving 0.00625% dinitrocresol growth and food consumption were slightly greater than the controls. In rats surviving on the diet containing 0.0125% dinitrocresol growth and food consumption were even greater than those of the previous group. With concentrations of 0.025%, growth was definitely inhibited in the survivors, although food consumption was greater than the controls. With concentrations of 0.05 and 0.1% rats failed to eat and died in two or three days.

At necropsy no gross changes were found. However, it is our impression that the skeletal tissues in some rats were stained yellow, particularly in rats on diets containing 0.0125 and 0.025% dinitrocresol. Blood serum from these rats appeared yellow as compared to serum from control rats. Histological examination of the visceral organs showed no pathologic changes that could be ascribed to the drug.

*Toxicity after local application.* Since dinitrocresol is used in the form of a spray, there remains the possibility that it may have an injurious effect upon the skin and eyes of those not protected. Therefore, these effects were studied after cutaneous application to the intact skin of rats and rabbits, and after instillation into the conjunctival sac of rabbits. A 2% aqueous solution of dinitrocresol was applied daily for 30 days to the depilated dorsal surface of 10 rats and to the depilated ventral surface of 6 rabbits. In all animals so treated there were no signs of local irritation or systemic effects. Rats were weighed at the beginning of the experiment and again on the last day of the experiment. Their weights were comparable to those of the untreated controls.

Since no local or systemic effects were demonstrable after cutaneous application to rats and rabbits, we decided to try this on humans. Accordingly, in two human subjects a 2% aqueous solution of the drug was applied daily to the shaved arm pits and to the anterior cubital surface of each arm for 30 days. Careful observations were made as to subjective and objective symptoms during the course of application. No local irritating effects were observed on either surfaces, and no systemic effects were apparent.

Instillation of 5 drops of a 1% aqueous solution of dinitrocresol into the conjunctival sac of 6 rabbits at half-hour intervals for 6 hours did not produce any visible signs of irritation either during instillation or 24 hours later.

*Effect on oxygen consumption, respiration, and circulation.* The action of dinitrocresol on oxygen consumption, respiration, and circulation was studied in dogs under barbital anesthesia. Oxygen consumption was recorded by the method described by Jackson (9), respiration by means of a pneumograph attached to a recording tambour, and blood pressure in the usual manner with a mercury manometer attached to the carotid artery through a cannula.

In one series of experiments the drug was injected intramuscularly in doses of 5 to 25 mgm. per kilogram in 10 dogs, and in another series it was injected intravenously in doses of 0.5 to 2 mgm. per kilogram in 6 dogs. In 8 dogs receiving 10 mgm. per kilogram intramuscularly the results were essentially the same as those shown in figure 1. Fifteen minutes after the injection oxygen consumption increased 100%, accompanied by a simultaneous increase in both rate and depth of respiration, with no apparent effect on blood pressure, heart rate, or body temperature. During the following 20 minutes oxygen consumption increased 200% followed by a further increase in rate and depth of respiration. Blood pressure remained unchanged, heart rate increased slightly, and body temperature increased 1.5°C. During the following 20 minute period there was a further

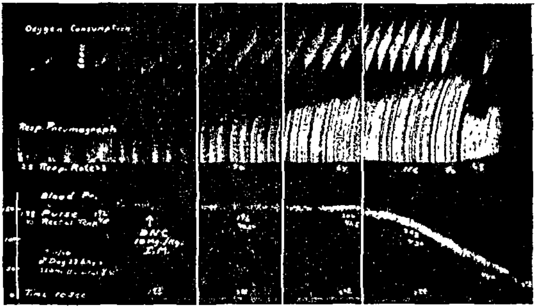

FIG. 1. EFFECT OF 3-5-DINITRO-O-CRESOL ON OXYGEN CONSUMPTION, RESPIRATION AND BLOOD PRESSURE AFTER INTRAMUSCULAR ADMINISTRATION

Dog, 23 6 kgm., ♂, barbital, 250 mgm per kilogram intravenously. From above downward record shows oxygen consumption, respiration, respiratory rate per minute, carotid arterial pressure, pulse rate per minute, rectal temperature in degrees centigrade, and time. At arrow 10 mgm of dinitrocresol per kilogram was injected intramuscularly. Death occurred at end of record.

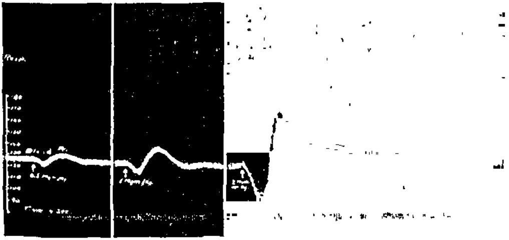

FIG. 2 EFFECT OF 3-5-DINITRO-O-CRESOL ON BLOOD PRESSURE AFTER INTRAVENOUS INJECTION

Dog 16 5 [illegible]

increase in oxygen consumption (300%), rate and depth of respiration, heart rate, and body temperature. Blood pressure, which had remained nearly constant up to this time, began to fall gradually and persistently, and continued to do so until the dog died. The changes shown in figure 1 are typical of those seen in all other dogs, with the exception that in some animals there was a slight rise in blood pressure shortly before the fall began. In 8 dogs receiving smaller or larger doses, the intensity and duration of action appeared to be proportional to the dose. None of these effects were observed with doses below 5 mgm. per kilogram.

After intravenous injection of 2 mgm. per kilogram a sudden increase in oxygen consumption, and rate and depth of respiration invariably occurred. Smaller doses had no particular effect on these functions. On blood pressure doses as low as 0.5 mgm. per kilogram caused a slight fall, followed by a rise, and a gradual return to the normal level. In doses of 1 or 2 mgm. per kilogram the intensity of effect on blood pressure appeared to be proportional to the dose. In figure 2 are shown the effects on blood pressure after the intravenous injection of 0.5, 1, and 2 mgm. per kilogram. Three mgm. per kilogram invariably proved fatal within 10 minutes, after profound stimulation of respiratory functions.

## SUMMARY AND CONCLUSIONS

Data are presented on the general and local effects of *3-5*-dinitro-*o*-cresol in experimental animals after various modes of administration.

The M.F.D. for rats was found to be 20 mgm. per kilogram after subcutaneous injection, and 30 mgm. per kilogram after oral administration.

Repeated subcutaneous administration of sublethal doses (15 mgm. per kilogram) in rats did not have any demonstrable effects on the animals' general condition or blood picture.

Chronic feeding experiments on white rats were made with *3-5*-dinitro-*o*-cresol in concentrations of from 0.00078 to 0.1% in the diet over a period of 105 days. Concentrations of 0.00078 to 0.00625% did not appreciably modify growth or food consumption of young rats maintained on these diets. In concentrations of 0.0125%, the drug was lethal to 60% of the animals, while growth and food consumption of the survivors were greater than that of the controls. First definite inhibition in growth occurred on the diet containing 0.025%, although food consumption of the survivors was greater than that of the controls. Concentrations of 0.05 and 0.1% were lethal.

Repeated injections of sublethal doses subcutaneously in rats, and repeated cutaneous application to the intact skin of experimental animals and humans failed to show any signs of irritation or inflammation. No indications have been found that absorption from these surfaces induces chronic poisoning, although it is well established that nitrated phenols are capable of being absorbed through the skin.

At necropsy, and on histopathological examination, the tissues of all rats receiving the drug subcutaneously or orally for 30 days or more failed to show any characteristic lesions that could be ascribed to the drug.

Intramuscular administration of doses as low as 10 mgm. per kilogram in dogs progressively increased oxygen consumption, rate and depth of respiration, and body temperature. Blood pressure was not materially affected until just before death. After the intravenous injection of 2 mgm. per kilogram the onset of stimulation of the respiratory functions was more rapid. On blood pressure, doses as low as 0.5 mgm. per kilogram produced a slight fall, followed by a rise and gradual return to normal. With doses of 1 and 2 mgm. per kilogram intravenously the intensity of effect on blood pressure appeared to be proportional to the dose.

Dinitrocresol in lethal concentrations after subcutaneous, intramuscular, oral or intravenous administration produces an acute toxemia characterized by increased rate and depth of respiration, hyperpyrexia, and by rapidly developing cardiac and respiratory failure, with *rigor mortis* setting in almost immediately following death.

I wish to acknowledge the assistance of the Department of Pathology for preparation of the microscopic sections and to Doctor A. J. Miller, Professor of Pathology, for the histological studies herein reported.

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# RELATIVE PHARMACOLOGICAL EFFECTS OF *2*-ALKYL-*3,4*-DIHYDROISOQUINOLINIUM CHLORIDES

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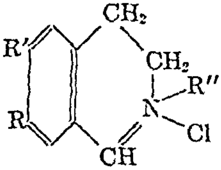
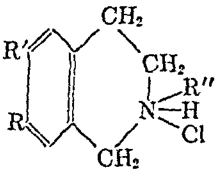
This report deals with the chemical and pharmacological properties of three homologous series of *2*-alkyl-*3,4*-dihydroisoquinolinium chlorides substituted in the *6* and *6,7* positions with hydroxy, methoxy and ethoxy groups. A previous paper was concerned with the corresponding derivatives of *1,2,3,4*-tetrahydroisoquinoline hydrochloride (1). The two groups of compounds are related structurally as follows:



*2*-Alkyl-*3,4*-dihydroisoquinolinium chloride



*2*-Alkyl-*1,2,3,4*-tetrahydroisoquinoline hydrochloride

*R* and *R′* are hydrogen, hydroxyl, methoxyl and ethoxyl. *R″* is an alkyl group. The above dihydro- compound is, of course, a quaternary ammonium salt, while the tetrahydro- compound is the hydrochloride of a tertiary amine.

The pharmacological properties of the *2*-methyl-*6,7*-dimethoxy-*3,4*-dihydroisoquinolinium chloride, known clinically as Lodal, were thoroughly studied in comparison with hydrastinine and cotarnine and the corresponding tetrahydroisoquinolines by Laidlaw (2). The *2*-methyl-*6,7*-dihydroxy-*3,4*-dihydro-derivative has been studied for its antispasmodic activity in frogs and rats (3).

Experimental. 1. *Chemistry.* The dihydroisoquinolinium chlorides were prepared by the action of silver chloride on the corresponding iodides in aqueous solution. The iodides were obtained by the addition of the alkyl iodide to a benzene solution of the alkoxy dihydroisoquinoline base. The preparation and properties of the dimethoxy iodides has been described by Buck and Ide (4), with the exception of the *2*-methyl compound described by Späth and Epstein and others (5). The other dihydroisoquinolinium chlorides were prepared in a similar manner from *3*-methoxy- (6), *3*-ethoxy- (7), and *3,4*-diethoxy- (7) -phenethylamines.

Demethylation to the corresponding hydroxy and dihydroxy compounds was carried out as previously described (4).

Difficulty was experienced in drying many of the hydrochlorides and melting points vary somewhat with conditions (dryness, etc.). Those given are therefore to be considered as approximate. Many of the salts show strong bluish-white fluorescence in aqueous or alcoholic solution.

*II. Toxicity.* The methods used for the determination of the LD 50 for albino mice were described previously (1).

TABLE 1

*Dihydroisoquinolinium chlorides*

| NO. | SUBSTITUENT GROUPS | APPEARANCE | M P. | FORMULA | ANALYSES | | | | SOLVENT RE-CRYST. | FLUO-RES-CENCE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Calcd. | | Found | | | |
| | | | | | C | H | C | H | | |
| | | | °C. (*corr.*) | | % | % | % | % | | |
| 765 | *2*-Methyl-*6,7*-dihydroxy (8)* | Light yellow powder (slender prisms) | 275° | $C_{10}H_{12}O_2NCl$ | 56.19 | 5.67 | 56.39 | 5.90 | M E† | – |
| 766 | *2*-Ethyl-*6,7*-dihydroxy (8) | Light yellow small glittering leaves | 204° | $C_{11}H_{14}O_2NCl$ | 58.01 | 6.20 | 58.19 | 6.20 | A E | – |
| 767 | *2*-*n*-Propyl-*6,7*-dihydroxy | Yellow-ocher clumps of thick plates | 232° | $C_{12}H_{16}O_2NCl$ | 59.60 | 6.67 | 59.97 | 6.79 | A E | – |
| 768 | *2*-Isopropyl-*6,7*-dihydroxy | Light yellow slender meshed needles | 232.5° | $C_{12}H_{16}O_2NCl$ | 59.60 | 6.67 | 59.73 | 6.82 | A E | – |
| 769 | *2*-*n*-Butyl-*6,7*-dihydroxy | Light yellow small silky leaves | 190.5° | $C_{13}H_{18}O_2NCl$ | 61.03 | 7.10 | 61.19 | 7.21 | A E | – |
| 770 | *2*-*n*-Amyl-*6,7*-dihydroxy | Yellow tiny shining leaves | 173.5° | $C_{14}H_{20}O_2NCl$ | 62.31 | 7.49 | 62.59 | 7.62 | A E | – |
| 771 | *2*-Isoamyl-*6,7*-dihydroxy | Dull yellow felted needles | 203° | $C_{14}H_{20}O_2NCl$ | 62.31 | 7.48 | 62.90 | 7.54 | A E | – |
| 750 | *2*-Methyl-*6,7*-dimethoxy (5)‡ | Light yellow tiny plates | 183°‖ | $C_{12}H_{16}O_2NCl$ | 59.60 | 6.67 | 59.76 | 6.89 | A E Æ | + |
| 751 | *2*-Ethyl-*6,7*-dimethoxy§ | Small white needles | 192° dec | $C_{13}H_{18}O_2NCl$ | 61.03 | 7.10 | 61.16 | 7.19 | A E Æ | + |
| 752 | *2*-*n*-Propyl-*6,7*-dimethoxy§ | Cream-colored clumps | 177° dec ‖ | $C_{14}H_{20}O_2NCl$ | 62.31 | 7.49 | 62.19 | 7.77 | A E | + |
| 753 | *2*-Isopropyl-*6,7*-dimethoxy | Small white powdery needles | 183° dec | $C_{14}H_{20}O_2NCl$ | 62.31 | 7.48 | 62.59 | 7.60 | A E | + |
| 754 | *2*-*n*-Butyl-*6,7*-dimethoxy | Pale yellow clumps of prisms | 169° dec ‖ | $C_{15}H_{22}O_2NCl$ | 63.46 | 7.82 | 63.72 | 8.09 | A E | + |
| 755 | *2*-*n*-Amyl-*6,7*-dimethoxy | Faint yellow small needle prisms | 143° | $C_{16}H_{24}O_2NCl$ | 64.51 | 8.13 | 64.59 | 8.35 | A E B | + |
| 756 | *2*-Isoamyl-*6,7*-dimethoxy | Light yellow prism aggregates | 160° dec ‖ | $C_{16}H_{24}O_2NCl$ | 64.51 | 8.13 | 64.62 | 8.31 | A E B | + |
| 748 | *2*-Methyl-*6,7*-diethoxy | Small leaves, part white, part yellow | 180° dec ‖ | $C_{14}H_{20}O_2NCl$ | 62.31 | 7.49 | 62.27 | 7.64 | A E | + |
| 746 | *2*-Methyl-*6*-methoxy¶ | Almost white small glittering leaves | 137°‖ | $C_{11}H_{14}ONCl$ | 62.39 | 6.67 | 62.72 | 6.69 | A E | – |
| 747 | *2*-Methyl-*6*-ethoxy | White felted needles | 150°‖ | $C_{12}H_{16}ONCl$ | 63.84 | 7.15 | 63.94 | 7.61 | Bu E | – |
| 749 | *2*-*n*-Butyl-*6*-ethoxy | Almost white rounded prisms | Soft 64°<br>Flow 76° | $C_{15}H_{22}ONCl$ | 67.27 | 8.29 | 67.33 | 8.42 | Bu E | – |

* Akabori prepared this from hydrastinine (M p., 276°) (9).

† A, absolute alcohol; Æ, ethyl acetate; Bu, butanol, B̈, benzene, E, ether, absolute; M, methanol

‡ This is Lodal (chloride) (10, 11)

§ Prepared in another way by Pyman (12)

¶ Iodide prepared by Gulland and Virden (13)

‖ Dried 12 hours at 100°C in vacuo; the others dried in vacuo at room temperature.

In general, the *2*-alkyl-*3,4*-dihydroisoquinolinium chlorides described in this paper were of the same order of toxicity as the *2*-alkyl-*1,2,3,4*-tetrahydroisoquinoline hydrochlorides (1). Likewise, the slopes of the dose-response curves were very steep.

It was found that the *6,7*-dihydroxy-*3,4*-dihydroisoquinolinium chlorides were less toxic than the corresponding *6,7*-dimethoxy compounds, resembling in this respect the *2*-alkyl-*1,2,3,4*-tetrahydroisoquinoline hydrochlorides (1). However, no progressive increase in toxicity accompanied the lengthening of the *2*-alkyl chain with either the dihydroxy or the dimethoxy compounds.

As with the *2*-alkyl-*1,2,3,4*-tetrahydroisoquinoline hydrochlorides (1), the presence of *6,7*-dihydroxy groups was associated with the production of sympathomimetic symptoms in albino mice. However, there seemed to be less pilomotor activity and more salivation with the *3,4*-dihydroisoquinolinium chlorides. In this connection, salivation was easier to detect with the latter compounds owing to the fact that they imparted to the saliva their brilliant yellow color. As evidenced by the color, they were also excreted in the urine. The *2*-alkyl-*6,7*-dihydroxy-*1,2,3,4*-tetrahydroisoquinoline hydrochlorides (1) did not produce tremors whereas fine tremors were generally observed with all of the *3,4*-dihydroisoquinolinium chlorides regardless of whether they contained hydroxyl groups or not. Methoxy and ethoxy derivatives were alike in both the *1,2,3,4*-tetrahydroisoquinoline hydrochlorides and in the *3,4*-dihydroisoquinolinium chlorides in their lack of sympathomimetic properties.

TABLE 2

*Relative toxicological effects of 2-alkyl-3,4-dihydroisoquinolinium chlorides on albino mice*

| NO. | SUBSTITUENT GROUPS | MOLECULAR WEIGHT | LD 50 | SLOPE | LIMITS OF ERROR | PILOMOTOR EFFECTS | EXOPHTHALMOS | SALIVATION | TREMORS |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm./kgm.* | | % | | | | |
| 765 | *2*-Methyl-*6,7*-dihydroxy | 213.6 | 120 | 47 | 96–104 | Slight | Slight | Moderate | |
| 766 | *2*-Ethyl-*6,7*-dihydroxy | 227.6 | 116 | 21 | 93–107 | Slight | Moderate, brief | | Fine |
| 767 | *2*-*n*-Propyl-*6,7*-dihydroxy | 241.6 | 134 | 19 | 94–107 | Slight | Moderate, brief | Moderate | Fine |
| 768 | *2*-Isopropyl-*6,7*-dihydroxy | 241.6 | 109 | 23 | 93–108 | Slight | Moderate, brief | Moderate | Fine |
| 769 | *2*-*n*-Butyl-*6,7*-dihydroxy | 255.6 | 179 | 18 | 93–106 | | Moderate, brief | Moderate | Fine |
| 770 | *2*-*n*-Amyl-*6,7*-dihydroxy | 269.6 | 129 | 34 | 95–105 | | Moderate, brief | Moderate | Fine |
| 771 | *2*-Isoamyl-*6,7*-dihydroxy | 269.6 | 170 | 13 | 88–113 | | Moderate, brief | Moderate | Fine |
| 750 | *2*-Methyl-*6,7*-dimethoxy | 241.6 | 92 | 9 | 90–111 | Slight | Marked, brief | | |
| 751 | *2*-Ethyl-*6,7*-dimethoxy | 255.6 | 99 | 15 | 92–109 | | Slight | | Fine |
| 752 | *2*-*n*-Propyl-*6,7*-dimethoxy | 269.6 | 73 | 24 | 93–108 | | | | Fine |
| 753 | *2*-Isopropyl-*6,7*-dimethoxy | 269.6 | 86 | 31 | 94–106 | | | | Fine |
| 754 | *2*-*n*-Butyl-*6,7*-dimethoxy | 283.7 | 126 | 25 | 95–105 | | | | Fine |
| 755 | *2*-*n*-Amyl-*6,7*-dimethoxy | 297.7 | 108 | 42 | 96–104 | | | | Fine |
| 756 | *2*-Isoamyl-*6,7*-dimethoxy | 297.7 | 130 | 34 | 96–104 | | | | Fine |
| 748 | *2*-Methyl-*6,7*-diethoxy | 269.6 | 124 | 17 | 94–106 | | | | |
| 746 | *2*-Methyl-*6*-methoxy | 211.6 | 166 | 33 | 96–104 | Slight | | | Coarse |
| 747 | *2*-Methyl-*6*-ethoxy | 225.6 | 184 | 39 | 95–105 | Slight | Slight | | |
| 749 | *2*-Butyl-*6*-ethoxy | 267.6 | | | | | | | |

A brief series of convulsive hops and symptoms of respiratory failure were observed shortly before death in all cases.

*III. Circulatory effects.* The general circulatory effects are summarized in table 3. The methods used were presented in a previous report (1).

Among the *6,7*-dihydroxy derivatives, the compounds with short carbon chains on the nitrogen were predominantly pressor while those with long chains were depressor. There was a gradual change from pure pressor to pure depressor with the lengthening of the carbon chain. Among the dimethoxy derivatives, only the *2*-methyl compound was pressor; the longer chain derivatives were depressor. It is of interest that whereas the *2*-methyl derivative was a pure short-acting pressor agent, as Laidlaw observed (2), the corresponding *6,7*-diethoxy derivative had long-lasting depressor effects. The *6*-methoxy and *6*-ethoxy derivatives had either biphasic depressor-pressor actions or were purely depressor. In general, it would be difficult to distinguish the qualitative blood pressure effects of the dihydroisoquinolinium salts from those of the corresponding tetrahydroisoquinolines (1) except in certain individual cases. The dihydroxy dihy-

TABLE 3

*Relative effects of 2-alkyl-3,4-dihydroisoquinolinium chlorides on blood pressure, respiration and pulse*

| NO. | SUBSTITUENT GROUPS | DOSE | | BLOOD PRESSURE | | PAIRED DOSE | EFFECT OF VAGOTOMY | EFFECT OF ATROPINE | EFFECT ON EPINEPHRINE | RESPIRATION | PULSE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Change | Duration | | | | | | |
| | | *mM/kgm.* | *mgm/kgm.* | *mm. Hg.* | *minutes* | | | | | | |
| 765 | *2*-Methyl-*6,7*-dihydroxy | .008 | 1.71 | p 12 | 3 | 0 | 0 | 0 | D | I | D |
| | | .064 | 13.66 | p 20 | 10 | | | | | | |
| 766 | *2*-Ethyl-*6,7*-dihydroxy | .008 | 1.82 | p 8 | 5 | 0 | 0 | 0 | S | 0 | D |
| | | .064 | 14.56 | p 35 | 12 | | | | | | |
| 767 | *2*-*n*-Propyl-*6,7*-dihydroxy | .008 | 1.93 | p 12 | 2 | 0 | 0 | 0 | S | 0 | 0 |
| | | .064 | 15.46 | p 18 | 5 | | | | | | |
| 768 | *2*-Isopropyl-*6,7*-dihydroxy | .008 | 1.93 | p 12 | 6 | 0 | 0 | 0 | S | 0 | 0 |
| | | .064 | 15.46 | d 10 p 12 | 1 5 | | | | | | |
| 769 | *2*-*n*-Butyl-*6,7*-dihydroxy | .008 | 2.04 | p 10 | 8 | 0 | 0 | 0 | S | 0 | 0 |
| | | .064 | 16.36 | d 30 p 13 | 1 10 | | | | | | |
| 770 | *2*-*n*-Amyl-*6,7*-dihydroxy | .008 | 2.16 | d 10 p 24 | 1 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | .064 | 17.24 | d 35 | 20 | | | | | | |
| 771 | *2*-Isoamyl-*6,7*-dihydroxy | .008 | 2.16 | d 10 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | .064 | 17.24 | d 40 | 1 | | | | | | |
| 750 | *2*-Methyl-*6,7*-dimethoxy | .004 | .96 | p 18 | 1 | 0 | 0 | D | D | I | D |
| | | .016 | 3.86 | p 110 | 5 | | | | | | |
| 751 | *2*-Ethyl-*6,7*-dimethoxy | .008 | 2.04 | p 17 d 10 | 1 5 | 0 | 0 | 0 | 0 | 0 | D |
| | | .064 | 16.39 | p 40 d 35 | 1 20 | | | | | | |
| 752 | *2*-*n*-Propyl-*6,7*-dimethoxy | .008 | 2.16 | d 10 | 1 | 0 | 0 | 0 | S | 0 | 0 |
| | | .064 | 17.24 | d 30 | 12 | | | | | | |
| 753 | *2*-Isopropyl-*6,7*-dimethoxy | .008 | 2.16 | d 10 | 2 | 0 | I | 0 | 0 | 0 | 0 |
| | | .064 | 17.24 | d 30 | 20 | | | | | | |
| 754 | *2*-*n*-Butyl-*6,7*-dimethoxy | .008 | 2.27 | d 25 | 2 | 0 | I | 0 | 0 | 0 | D |
| | | .032 | 9.08 | d 43 | 20 | | | | | | |
| 755 | *2*-*n*-Amyl-*6,7*-dimethoxy | .008 | 2.39 | d 30 | 2 | 0 | I | 0 | 0 | 0 | D |
| | | .032 | 9.52 | d 32 | 20 | | | | | | |
| 756 | *2*-Isoamyl-*6,7*-dimethoxy | .008 | 2.39 | d 25 | 2 | 0 | I | 0 | 0 | 0 | D |
| | | .064 | 19.04 | d 70 | 10 | | | | | | |
| 748 | *2*-Methyl-*6,7*-diethoxy | .008 | 2.16 | d 14 | 20 | 0 | 0 | 0 | S | 0 | 0 |
| | | .032 | 8.64 | d 20 | 60 | | | | | | |
| 746 | *2*-Methyl-*6*-methoxy | .008 | 1.69 | d 49 p 46 | 1 2 | 0 | a | 0 | S | I | D |
| | | .032 | 6.76 | d 40 p 62 | 1 9 | | | | | | |
| 747 | *2*-Methyl-*6*-ethoxy | .008 | 1.80 | d 18 p 20 | 1 1 | 0 | a | 0 | S | I | D |
| | | .032 | 7.22 | d 18 p 55 | 1 4 | | | | | | |
| 749 | *2*-*n*-Butyl-*6*-ethoxy | .004 | 1.07 | d 47 | 1 | 0 | 0 | 0 | S | 0 | D |
| | | .032 | 8.56 | d 24 | 15 | | | | | | |

*d*, depressor, *a*, abolished depressor; *p*, pressor; *D*, decrease; *I*, increase; *S*, sensitized; 0, no change.
Each compound was tested on two dogs

droisoquinolinium chlorides were somewhat shorter-acting and less potent pressors than the tetrahydro analogs. The *2*-methyl-*6*,*7*-dimethoxy-*3*,*4*-dihydroisoquinolinium salt (Lodal) was pressor whereas the corresponding tetrahydroisoquinoline derivative was depressor. Among the depressor agents, there was little to distinguish among the analogs except in the length of action with large doses.

None of the dihydroisoquinolinium salts showed tachyphylaxis as evidenced by the paired dose responses. Vagotomy had no effect on the responses of the pressor compounds but it enhanced the response of several of the depressor agents, apparently indicating a central vagus depressant action. The initial depressor phase of several of the biphasic compounds was abolished by vagotomy, apparently indicating a central vagus stimulating action. Atropinization usually had no influence on the response of the compounds.

TABLE 4

*Effects of 2-alkyl-3,4-dihydroisoquinolinium chlorides on tone of isolated smooth muscle*

| NO. | SUBSTITUENT GROUPS | CONCENTRATION IN BATH | RABBIT INTESTINE | RABBIT UTERUS | GUINEA PIG UTERUS | NUMBER OF TESTS |
|---|---|---|---|---|---|---|
| | | *milli-molar* | | | | |
| 765 | *2*-Methyl-*6*,*7*-dihydroxy | 0.1–0.2 | + | + | + | 3 |
| 766 | *2*-Ethyl-*6*,*7*-dihydroxy | 0.1–0.2 | + + | + | + | 3 |
| 767 | *2*-*n*-Propyl-*6*,*7*-dihydroxy | 0.1–0.2 | + | + + | + | 3 |
| 768 | *2*-Isopropyl-*6*,*7*-dihydroxy | 0.1–0.2 | + + | + | + | 5 |
| 769 | *2*-*n*-Butyl-*6*,*7*-dihydroxy | 0.1–0.2 | + | + | + + | 4 |
| 770 | *2*-*n*-Amyl-*6*,*7*-dihydroxy | 0.1–0.2 | + + | + + | + + | 4 |
| 771 | *2*-Isoamyl-*6*,*7*-dihydroxy | 0.1–0.2 | + | + + | + + | 5 |
| 750 | *2*-Methyl-*6*,*7*-dimethoxy | 0.2–0.4 | − | + | + | 5 |
| 751 | *2*-Ethyl-*6*,*7*-dimethoxy | 0.2–0.4 | − − | + | + | 5 |
| 752 | *2*-*n*-Propyl-*6*,*7*-dimethoxy | 0.2–0.4 | − − | + + | + + | 5 |
| 753 | *2*-Isopropyl-*6*,*7*-dimethoxy | 0.1–0.2 | (+ −) | + + | + + | 4 |
| 754 | *2*-*n*-Butyl-*6*,*7*-dimethoxy | 0.1–0.2 | (+ −) | + + | + + | 4 |
| 755 | *2*-*n*-Amyl-*6*,*7*-dimethoxy | 0.1–0.2 | (+ −) | + + | + + | 5 |
| 756 | *2*-Isoamyl-*6*,*7*-dimethoxy | 0.1–0.2 | (+ −) | + + | + + | 5 |
| 748 | *2*-Methyl-*6*,*7*-diethoxy | 0.1–0.2 | − | + + | + + | 4 |
| 746 | *2*-Methyl-*6*-methoxy | 0.2–0.4 | (+ −) | + | + + | 5 |
| 747 | *2*-Methyl-*6*-ethoxy | 0.2–0.4 | (+ −) | + | + + | 4 |
| 749 | *2*-Butyl-*6*-ethoxy | 0.1–0.2 | (+ −) | + + | + + | 4 |

−, moderate decrease; − −, marked decrease; +, moderate increase; + +, marked increase; (+ −), increase or decrease.

The epinephrine response was usually sensitized by the pressor substances whereas it was usually unaffected by the depressor compounds. The depressor agents among the dihydroisoquinolinium salts did not have the epinephrine-inhibiting properties of the analogous tetrahydroisoquinoline derivatives (1) or the adrenolytic properties of the unsubstituted homologous tetrahydroisoquinoline derivatives (14).

Respiration was usually unaffected by these compounds.

The pulse rate was usually unchanged, but was slowed by an occasional compound.

*IV. Smooth muscle effects.* The relative qualitative effects of the present series of isoquinolines on smooth muscle as observed on isolated tissues (1) are recorded in table 4.

It was observed that the dihydroxy derivatives consistently stimulated the intestine; several of the dimethoxy derivatives relaxed this tissue and the remainder had mixed stimulating and depressing actions. The corresponding tetrahydro- analogs were consistent depressors of the intestine (1). All of the *3*,*4*-dihydroisoquinolinium salts as well as their tetrahydroisoquinoline analogs (1) stimulated both the rabbit and guinea pig uteri.

## SUMMARY

The chemical and pharmacological properties of three homologous series of *2*-alkyl-*3*,*4*-dihydroisoquinolinium chlorides substituted in the *6* and *6*,*7* positions with hydroxy, methoxy and ethoxy groups have been studied. These *2*-alkyl-*3*,*4*-dihydroisoquinolinium chlorides were of the same order of toxicity as the corresponding *2*-alkyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline hydrochlorides.

Among the dihydroxy derivatives, lengthening of the carbon chain attached to the nitrogen converted the pure pressors into depressors. With the exception of the pressor *2*-methyl-*6*,*7*-dimethoxy derivative (Lodal), the methoxy and ethoxy derivatives were predominantly depressors or biphasic depressor-pressors.

No evidence of tachyphylaxis was obtained.

Vagotomy had no effect on the pressors; it sensitized some of the depressors; and abolished the depressor phase of some of the mixed-acting compounds. Atropine usually had no effect.

The epinephrine response was usually enhanced by the pressors but unaffected by the other compounds.

The respiration and pulse rate were usually unaffected.

The dihydroxy derivatives stimulated the intestine while the remaining derivatives either stimulated or depressed. The rabbit and guinea pig uteri were stimulated by all the compounds.

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# RELATIVE PHARMACOLOGICAL EFFECTS OF *3*-METHYL-*3,4*-DIHYDRO- AND *3*-METHYL-*1,2,3,4*-TETRAHYDRO-ISOQUINOLINE DERIVATIVES

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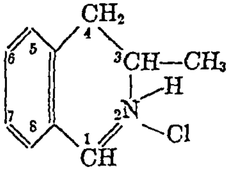
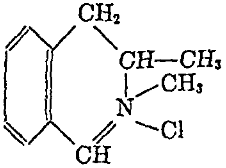
This report deals with the general toxicological, circulatory and smooth muscle effects of a series of *3*-methyl-*3,4*-dihydro- and *3*-methyl-*1,2,3,4*-tetrahydroisoquinoline derivatives, substituted in the *6,7* positions by dihydroxy, methylenedioxy or dimethoxy groups, some in addition carrying *2*-methyl or *2*-dimethyl groups. These isoquinoline derivatives may be considered to be related to β-phenylisopropylamine, as shown by the following formulae, the *6,7* positions being occupied by two hydroxy groups, two methoxy groups or one methylenedioxy group:



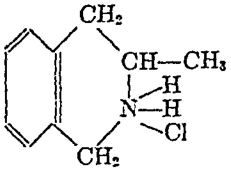
I

*3*-Methyl-*3,4*-dihydro-isoquinoline hydrochloride



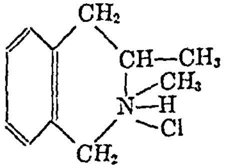
II

*2,3*-Dimethyl-*3,4*-dihydro-isoquinolinium chloride



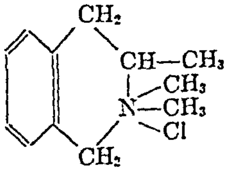
III

*3*-Methyl-*1,2,3,4*-tetrahydro-isoquinoline hydrochloride



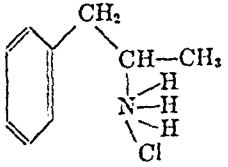
IV

*2,3*-Dimethyl-*1,2,3,4*-tetrahydro-isoquinoline hydrochloride



VI

*2,2,3*-Trimethyl-*1,2,3,4*-tetrahydro-isoquinolinium chloride



V

β-Phenylisopropylamine hydrochloride

Compounds of type III may be regarded as the hydrochlorides of secondary amines; compounds of types I and IV as the hydrochlorides of tertiary amines; and compounds of types II and V as quaternary salts.

The series, synthesized by Ide and Buck (1), was made in order to study the influence of the *3*-methyl group on the pharmacological properties of isoquinoline derivatives. We have previously reported on the analogous isoquinoline derivatives *unsubstituted in the 3 position* (2, 3 & 4).

EXPERIMENTAL. *I. Toxicity.* The relative toxicity values and symptoms in albino mice obtained by methods previously described (6) are recorded in table 1. The secondary tetrahydroisoquinoline compounds were least toxic and the quaternary dihydro- and tetrahydroisoquinolinium chlorides were most toxic, the substituents on the *6,7* positions having much less effect on relative toxicities than the substituents on the nitrogen. The hydroxy derivatives were least toxic among the secondary and tertiary amines but not among the quaternary salts.

TABLE 1

*Relative toxicity and symptoms of 3-methyl-3,4-dihydro- and 3-methyl-1,2,3,4-tetrahydroisoquinoline derivatives*

| NO. | COMPOUND TYPE | SUBSTITUENT GROUP IN *6,7* POSITION | MOLECULAR WEIGHT | LD 50 | SLOPE | LIMITS OF ERROR | PILOMOTOR EFFECTS | EXOPHTHALMOS | SALIVATION | TREMORS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *mgm./kgm.* | | *per cent* | | | | |
| 898 | III | Dihydroxy | 215.6 | 460 | 15 | 91–119 | Moderate | Moderate | Occasional | |
| 787 | III | Methylenedioxy | 227.6 | 182 | 40 | 97–103 | | Pronounced | | Intermittent |
| 795 | III | Dimethoxy | 243.6 | 272 | 53 | 97–104 | | Brief | | Coarse |
| 896 | I | Dihydroxy | 213.6 | 169 | 17 | 94–107 | | | | |
| 784 | I | Methylenedioxy | 225.6 | 75 | 40 | 97–103 | Slight | Moderate | | |
| 792 | I | Dimethoxy | 241.6 | 120 | 18 | 93–107 | | Brief | | Coarse |
| 899 | IV | Dihydroxy | 229.6 | 127 | 15 | 91–109 | Slight, brief | Slight, brief | | |
| 788 | IV | Methylenedioxy | 241.6 | 80 | 15 | 90–111 | Slight | Pronounced | | |
| 797 | IV | Dimethoxy | 271.6 | 52 | 40 | 95–105 | | Brief | | Coarse |
| 897 | II | Dihydroxy | 227.6 | 67 | 17 | 93–107 | | Slight, brief | | |
| 785 | II | Methylenedioxy | 239.6 | 80 | 20 | 94–106 | | Moderate | | |
| 793 | II | Dimethoxy | 255.6 | 30 | 29 | 96–104 | | Brief | | |
| 900 | V | Dihydroxy | 243.6 | 11 | 8 | 85–118 | Moderate, brief | Slight, brief | | |
| 790 | V | Methylenedioxy | 255.6 | 28 | 29 | 96–105 | | Pronounced | | |
| 789 | V | Dimethoxy | 271.6 | 37 | 45 | 97–103 | Slight | Brief | | Occasional, coarse |

Roman numerals refer to compounds the formulae of which are given in the introduction.

However, the symptoms observed in the mice were more closely associated with the *6,7* position substituents than with the nitrogen substituents. Although pilomotor effects were observed only with occasional compounds, exophthalmos was most pronounced with the methylenedioxy derivatives while tremors were noted mostly with the dimethoxy compounds.

A brief period of respiratory distress and short convulsive hops preceded death in all cases; however, the convulsions appeared to be less severe and of shorter duration with the quaternary compounds than with the tertiary. Thus these *3*-methyl substituted compounds also appear to follow, to some extent, Laidlaw's generalization (5) that isoquinoline compounds substituted in the *6,7* positions with methoxy or methylene dioxy groups were convulsants when the nitrogen was trivalent, but were devoid of this effect when the nitrogen was pentavalent.

TABLE 2

*Relative effects of 3-methyl-3,4-dihydro- and 3-methyl-1,2,3,4-tetrahydroisoquinoline derivatives on circulation, respiration and pulse*

| NO. | COMPOUND TYPE | SUBSTITUENT GROUP IN 6,7 POSITION | DOSE | | BLOOD PRESSURE | | PAIRED DOSE | EFFECT OF VAGOTOMY | EFFECT OF ATROPINE | EFFECT ON EPINEPHRINE | RESPIRATION | PULSE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mM./kgm.* | *mgm./kgm.* | Change *mm. Hg* | Duration *minutes* | | | | | | |
| 898 | III | Dihydroxy | .008 | 1.72 | d 10 p 10 | <1 1 | 0 | 0 | 0 | 0 | I | D |
| | | | .064 | 13.80 | d 20 p 30 | <1 10 | | | | | | |
| 787 | III | Methylenedioxy | .008 | 1.82 | d 11 p 13 | <1 10 | d | 0 | 0 | D | I | D |
| | | | .064 | 14.56 | d 50 | 60 | | | | | | |
| 795 | III | Dimethoxy | .004 | .97 | d 5 p 15 | <1 5 | 0 | a | 0 | S | I | 0 |
| | | | .032 | 7.78 | d 30 p 10 | <1 5 | | | | | | |
| 896 | I | Dihydroxy | .008 | 1.71 | d 6 p 5 | <1 5 | 0 | 0 | 0 | S | I | 0 |
| | | | .064 | 13.68 | d 19 p 10 | <1 10 | | | | | | |
| 784 | I | Methylenedioxy | .008 | 1.80 | d 26 p 28 | <1 10 | 0 | a | 0 | S | I | D |
| | | | .032 | 7.22 | d 25 p 53 | <1 25 | | | | | | |
| 792 | I | Dimethoxy | .008 | 1.93 | d 20 p 11 | <1 5 | d | 0 | 0 | S | I | 0 |
| | | | .032 | 7.72 | d 32 | 15 | | | | | | |
| 899 | IV | Dihydroxy | .008 | 1.84 | d 8 p 10 | <1 2 | 0 | a | 0 | 0 | I | D |
| | | | .064 | 14.68 | d 13 p 44 | <1 8 | | | | | | |
| 788 | IV | Methylenedioxy | .008 | 1.93 | d 47 p 59 | <1 5 | 0 | 0 | 0 | D | I | D |
| | | | .064 | 15.46 | d 62 p 50 | <1 15 | | | | | | |
| 797 | IV | Dimethoxy | .004 | 1.03 | d 32 p 18 | <1 5 | d | 0 | 0 | S | I | D |
| | | | .032 | 8.24 | d 52 | 10 | | | | | | |
| 897 | II | Dihydroxy | .008 | 1.82 | d 8 p 16 | <1 1 | 0 | a | 0 | 0 | I | D |
| | | | .064 | 14.56 | d 54 p 25 | 1 5 | | | | | | |
| 785 | II | Methylenedioxy | .008 | 1.92 | p 38 | 3 | 0 | 0 | 0 | S | I | D |
| | | | .032 | 7.66 | p 97 | 9 | | | | | | |
| 793 | II | Dimethoxy | .002 | .51 | p 42 | 2 | 0 | D | D | S | I | 0 |
| | | | .008 | 2.04 | p125 | 5 | | | | | | |
| 900 | V | Dihydroxy | .002 | .49 | p 35 | 1 | 0 | D | 0 | S | I | D |
| | | | .032 | 7.78 | p121 | 5 | | | | | | |
| 790 | V | Methylenedioxy | .0005 | .13 | p 40 | 1 | 0 | 0 | D | S | I | D |
| | | | .004 | 1.02 | p145 | 5 | | | | | | |
| 798 | V | Dimethoxy | .001 | .27 | p 45 | 2 | 0 | D | D | S | I | D |
| | | | .008 | 2.18 | p140 | 6 | | | | | | |

Roman numerals refer to compounds the formulae of which are given in the introduction. *d*, depressor; *a*, abolished depressor; *p*, pressor; *D*, decrease; *I*, increase; *S*, sensitized; 0, no change. Each compound was tested on two dogs.

*II. Circulatory effects.* The general circulatory effects, recorded by methods previosly described (6), are summarized in table 2. The substituents on the *6,7* positions (dihydroxy, methylenedioxy, and dimethoxy) had no outstanding qualitative or quantitative influence on the blood pressure effects of the *3*-methyl derivatives. Likewise there was little difference between the corresponding dihydro- and tetrahydroisoquinoline derivatives. However, the direction of the response was strongly influenced by the substituents on the nitrogen: the secondary and tertiary amines had diphasic depressor-pressor, or, with large doses, purely depressor effects; the quaternary ammonium salts, with the exception of the *2,3*-dimethyl-*6,7*-dihydroxy-*3,4*-dihydroisoquinolinium chloride, were purely pressor. This result agreed with a previous finding that the quaternary grouping conferred pressor potency (2). Apparently the *3*-methyl substituent had little weight in influencing the response to the methylenedioxy and dimethoxy derivatives, but it introduced an initial depressor phase into the action of the dihydroxy derivatives, since the corresponding derivatives with the *3*-position unsubstituted were purely pressor (3,6).

TABLE 3

*Effect of 3-methyl-3,4-dihydro- and 3-methyl-1,2,3,4-tetrahydroisoquinoline derivatives on tone of isolated smooth muscles*

| NO. | COMPOUND TYPE | SUBSTITUENT GROUP IN *6,7* POSITON | CONCENTRATION IN BATH | RABBIT INTESTINE | RABBIT UTERUS | GUINEA PIG UTERUS | NUMBER OF TESTS |
|---|---|---|---|---|---|---|---|
| | | | *milli-molar* | | | | |
| 898 | III | Dihydroxy | 0.1–0.4 | (+ −) | + | + | 4 |
| 787 | III | Methylenedioxy | 0.1–0.2 | − − | + + | + | 3 |
| 795 | III | Dimethoxy | 0.1–0.2 | − | + + | + | 2 |
| 896 | I | Dihydroxy | 0.1–0.4 | − | + | + | 3 |
| 784 | I | Methylenedioxy | 0.1–0.2 | − − | + + | + | 3 |
| 792 | I | Dimethoxy | 0.1–0.2 | − − | + + | + | 2 |
| 899 | IV | Dihydroxy | 0.1–0 2 | − | + | + | 2 |
| 788 | IV | Methylenedioxy | 0.1–0.2 | − − | + | + + | 2 |
| 797 | IV | Dimethoxy | 0.1–0.2 | − − | + | + + | 2 |
| 897 | II | Dihydroxy | 0.1–0.4 | (+ −) | + | + | 4 |
| 785 | II | Methylenedioxy | 0.1–0.4 | − | + + | + + | 3 |
| 793 | II | Dimethoxy | 0.1–0.2 | − | + | + | 2 |
| 900 | V | Dihydroxy | 0.1–0.2 | (+ −) | + | + | 3 |
| 790 | V | Methylenedioxy | 0.1–0.4 | − | + | + + | 3 |
| 798 | V | Dimethoxy | 0.1–0.2 | − − | + + | + | 2 |

Roman numerals refer to compounds the formulae of which are given in the introduction.

−, moderate decrease; − −, marked decrease; +, moderate increase; + +, marked increase; (+ −), increase or decrease.

There was little evidence of tachyphylaxis except among the compounds which were predominantly depressor in the large dose range; in such cases the paired doses were depressor. The infrequent occurrence of tachyphylaxis among the *3*-methyl isoquinoline derivatives is striking in view of this well-known property of β-phenylisopropylamine (7). Vagotomy decreased the pressor effect of an occasional quaternary ammonium salt; it abolished the depressor phase of some of the biphasic depressor-pressor compounds, but had no effect with other similar compounds. Atropine inhibited the pressor effect of several of the quaternary salts but had no influence on the response to the remaining compounds. The epinephrine response was usually enhanced; three of the dihydroxy derivatives had no effect while two of the methylenedioxy derivatives inhibited the response. There was consistently an initial respiratory stimulation of short duration with all the compounds. The pulse rate was usually decreased.

*III. Smooth muscle effects.* The qualitative action of the compounds on isolated smooth muscle, studied by methods previously recorded (6), is summarized in table 3. These *3*-methyl-substituted isoquinoline derivatives usually relaxed the intestine. An occasional dihydroxy derivative had mixed effects, either stimulating or relaxing or both. All of the compounds consistently stimulated both the rabbit and guinea pig uteri, reactions characteristic of the isoquinoline type (2,3,6).

## SUMMARY

A series of *3*-methyl-*3,4*-dihydro- and *3*-methyl-*1,2,3,4*-tetrahydroisoquinoline derivatives, substituted in the *6,7* positions with dihydroxy, methylenedioxy and dimethoxy groups, were studied for their relative toxicological, circulatory and smooth muscle effects.

The secondary tetrahydroisoquinoline hydrochlorides were least toxic while the quaternary salts were most toxic. The substituents on the *6,7* positions had much less influence on toxicities than the substituents on the nitrogen. Exophthalmos was most pronounced with the methylenedioxy derivatives while tremors were observed only with the dimethoxy derivatives.

The substituents in the *6,7* positions had relatively little influence on the blood pressure response indicating that the pressor potency of the dihydroxy groups has been nullified by the *3*-methyl substituent. The quaterary ammonium salts were usually pressor while the secondary and tertiary compounds were diphasic depressor-pressors.

There was little evidence of tachyphylaxis in contrast to the results usually obtained with β-phenylisopropylamines.

Vagotomy and atropine inhibited the pressor response of some of the pressors and vagotomy abolished the depressor phase of some of the mixed-acting compounds.

The epinephrine effect was usually potentiated. Respiration was usually initially stimulated. The pulse rate was usually decreased. The intestine was usually relaxed and the rabbit and guinea pig uteri were stimulated.

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# RELATIVE PHARMACOLOGICAL EFFECTS OF *1*-METHYL-*3*,*4*-DIHYDRO- AND *1*-METHYL-*1*,*2*,*3*,*4*-TETRAHYDROISOQUINOLINE DERIVATIVES

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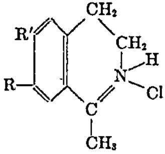
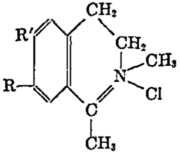
Received for publication August 8, 1942

This report deals with the chemistry and the relative pharmacological effects of a series of *1*-methyl-*3*,*4*-dihydro- and *1*-methyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline derivatives substituted in the *6* and *6*,*7* positions with hydroxy, methoxy and ethoxy groups. The structural relations are as follows:



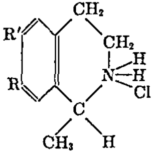
I

*1*-Methyl-*3*,*4*-dihydroisoquinoline hydrochloride



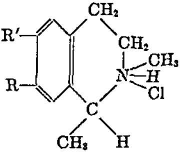
II

*1*,*2*-Dimethyl-*3*,*4*-dihydroisoquinolinium chloride



III

*1*-Methyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline hydrochloride



IV

*1*,*2*-Dimethyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline hydrochloride

In the above, *R* and *R′* represent hydrogen, hydroxyl, methoxyl and ethoxyl. Compounds of type III may be considered as secondary amine hydrochlorides; types I and IV are tertiary amine hydrochlorides; and type II compounds are quaternary ammonium salts.

The natural alkaloid, carnegine, which is the *1*,*2*-dimethyl-*6*,*7*-dimethoxy-*1*,*2*,*3*,*4*-tetrahydroisoquinoline, was found to increase the reflex excitability of frogs (1). The pharmacological properties of analogous dihydro- and tetrahydroisoquinoline derivatives with the 1 position unsubstituted have been reported previously (2, 3).

EXPERIMENTAL. *I. Chemistry.* The phenethylamines (4, 5) were acetylated by the method of Späth and Polgar (6) and the acetyl derivative then cyclized with phosphorus oxychloride (7). Reduction of the dihydroisoquinolines to the tetrahydro compounds was carried out by means of zinc and sulfuric acid. The methiodides were reduced in a similar manner (7).

TABLE 1

*1-Methyl-3,4-dihydroisoquinoline hydrochlorides, 1,2-dimethyl-3,4-dihydroisoquinolinium chlorides, and 1-methyl-1,2,3,4-tetrahydroisoquinoline hydrochorides*

| NO. | COMPOUND TYPE | SUBSTITUENT GROUPS | APPEARANCE | M.P. | FORMULA | ANALYSES |  |  |  | SOLVENT RE-CRYST. |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  | Calcd. |  | Found |  |  |
|  |  |  |  |  |  | C | H | C | H |  |
|  |  |  |  | °C. *(corr.)* |  | % | % | % | % |  |
| 734 | III | 6,7-Dihydroxy | Tiny, powdery aggregates | 230* | $C_{10}H_{12}O_2NCl$ | 55.67 | 6.55 | 55.95 | 6.62 | M E |
| 732 | III | 6,7-Dimethoxy | White, small tufts of prisms | 192* | $C_{12}H_{16}O_2NCl$ | 59.11 | 7.45 | 59.24 | 7.72 | A Æ |
| 731 | III | 6-Ethoxy | White, tiny tufts of flat prisms | 247* | $C_{12}H_{16}ONCl$ | 63.27 | 7.97 | 63.49 | 8.08 | M Æ |
| 733 | III | 6-Ethoxy-7-methoxy | White, felted flat needles | 253* | $C_{13}H_{18}O_2NCl$ | 60.56 | 7.83 | 60.82 | 8.13 | aHCl |
| 740 | IV | 2-Methyl | Aggregates of tiny stout prisms | 193* | $C_{11}H_{14}NCl$ | 66.81 | 8.16 | 67.03 | 8.37 | A Æ |
| 744 | IV | 2-Methyl-6,7-dihydroxy | Dull white clumps of tiny spindles | 207-209** | $C_{11}H_{14}O_2NCl$ | 57.49 | 7.03 | 57.44 | 7.22 | M E |
| 742 | IV | 2-Methyl-6,7-dimethoxy | White tiny nodules | 211-212* | $C_{13}H_{18}O_2NCl$ | 60.56 | 7.83 | 60.71 | 8.05 | A Æ |
| 741 | IV | 2-Methyl-6-ethoxy | White, tiny aggregates of leafy plates | 191.5* | $C_{13}H_{18}ONCl$ | 64.57 | 8.34 | 64.79 | 8.49 | A Æ |
| 743 | IV | 2-Methyl-6-ethoxy-7-methoxy | White aggregates of tiny plates | 212.5* | $C_{14}H_{20}O_2NCl$ | 61.85 | 8.16 | 62.00 | 8.18 | A Æ |
| 730 | I | 6,7-Dihydroxy | Light yellow small flat prisms | 254* | $C_{10}H_{14}O_2NCl$ | 56.19 | 5.67 | 56.29 | 5.87 | A E |
| 728 | I | 6,7-Dimethoxy | White, small, stout prisms | 205* dec. | $C_{12}H_{18}O_2NCl$ | 59.60 | 6.67 | 59.69 | 6.79 | A Æ |
| 727 | I | 6-Ethoxy | White, bulky powder (small needles) | 197** | $C_{12}H_{18}ONCl$ | 63.84 | 7.15 | 64.11 | 7.22 | A E |
| 729 | I | 6-Ethoxy-7-methoxy | White aggregates of small prisms | 234-235** dec. | $C_{13}H_{20}O_2NCl$ | 61.03 | 7.10 | 61.32 | 7.31 | A Æ |
| 739 | II | 2-Methyl-6,7-dihydroxy | Light yellow, felted, tiny needles | 239-240** dec. | $C_{11}H_{16}O_2NCl$ | 58.01 | 6.20 | 58.21 | 6.31 | M E |
| 736 | II | 2-Methyl-6,7-dimethoxy | Faint yellow, small, flat prisms | 181** dec | $C_{13}H_{20}O_2NCl$ | 61.03 | 7.10 | 61.10 | 7.24 | A Æ |
| 735 | II | 2-Methyl-6-ethoxy | White, felted, tiny needles | 203** dec. | $C_{13}H_{20}ONCl$ | 65.11 | 7.57 | 65.33 | 7.78 | A E |
| 738 | II | 2-Methyl-6-ethoxy-7-methoxy | White clumps of tiny crystals | 197** dec | $C_{14}H_{22}O_2NCl$ | 62.31 | 7.48 | 62.55 | 7.57 | A E |

*A*, ethanol; *a*, aqueous; *Æ*, ethyl acetate; *E*, ether; *M*, methanol.

* *Dried 12 hours at 100° in vacuo.*

Roman numerals refer to compounds the formulae of which are given in the introduction.

Demethylation of the dimethoxy compounds was carried out as described by Buck and Ide (7).

The conversion of iodides into chlorides was done in the usual manner by means of silver chloride.

Methiodides were prepared by allowing a benzene solution of the base to stand at room temperature with a slight excess of methyl iodide (7).

Many of the hydrochlorides are difficult to dry and some are hygroscopic. Melting points vary with the dryness, subdivision, etc., and those given must be regarded as approximate. Fluorescence (strong bluish-white) is shown only by compounds 728 and 729. The white color of most of the dihydroisoquinolinium chlorides is noteworthy.

*Individual preparations. 734.* The hydrobromide was described by Schöpf and Bayerle (12). The hydrochloride was prepared from 732 by demethylation.

*732.* Compound *728* was reduced. *732* is the *dl*-salsolidine hydrochloride of Späth and Dengel (11).

*731.* Compound *727* was reduced.

*733.* Compound *729* was reduced.

*740.* The base was prepared according to Freund and Bode (10) and an ether solution treated with hydrogen chloride.

*744.* *dl*-Carnegine hydrochloride (*742*) was demethylated. Schopf and Bayerle (12) prepared the picrate in another way.

*742.* The methiodide of the base of *728* was treated with silver chloride. Compound *742* is *dl*-carnegine hydrochloride, which was synthesized by Späth (9) in a similar manner.

*741.* The methiodide of *727* was reduced.

*743.* The methiodide of *729* was reduced.

*730.* This compound was prepared by demethylation of *728*.

*728.* Acetyl homoveratrylamine was cyclized and the hydrochloride prepared from the base by passing hydrogen chloride into the ether solution. The base has been described by Spath and Polgar (6), and the hydrochloride (different synthesis) by Kaufmann and Radošević, (m.p., 200°) (8).

*727.* *3*-Ethoxyphenethylamine (5) was acetylated, the product cyclized, and the hydrochloride prepared as with *728*.

*729.* *3*-Ethoxy-*4*-methoxyphenethylamine (5) was acetylated and cyclized and the base converted into the hydrochloride (Cf. *728*).

*739.* Compound *736* was demethylated.

*736.* The methiodide of *728* (9) was treated with silver chloride.

*735.* The methiodide of *727* was converted into the chloride.

*738.* The methiodide of *729* was treated with silver chloride.

*II. Toxicity.* The methods used for the determination of the LD 50 in albino mice were described previously (13). In general, tertiary compounds of type III, i.e., *1*-methyl-*1*,*2*,*3*,*4*-tetrahydroisoquinoline hydrochlorides, were less toxic than their corresponding homologues while quaternary compounds of type IV were more toxic. The *6*-ethoxy-*7*-methoxy-*1*,*2*-dimethyl-*1*,*2*,*3*,*4*-tetrahydroisoquinolinium chloride was found to have the exceptionally low LD 50 value of 11 mgm. per kgm.

The *6*,*7*-dihydroxy compounds were generally less toxic than the other members of their respective group types while the *6*-ethoxy-*7*-methoxy compounds were generally more toxic.

Signs of sympathomimetic activity such as exophthalmos and pilomotor effects were frequently found to be associated with the *6*,*7*-dihydroxy compounds which were generally found to possess pressor properties. These symptoms however were not particularly prominent and were absent in the case of *1*-methyl-*6*,*7*-dihydroxy-*3*,*4*-dihydroisoquinoline hydrochloride. With the exception of most of the *6,7*-dihydroxy compounds tremors were commonly produced. Brief convulsive hops and respiratory distress preceded death in all cases.

*III. Circulatory effects.* The relative circulatory effects, as recorded kymographically (13), are summarized in table 3. The *6,7*-dihydroxy derivatives of both the dihydro- and tetrahydro-isoquinoline compounds were predominantly pressor as were the corresponding isoquinoline derivatives unsubstituted in the *1* position (2, 3). The methoxy and ethoxy derivatives had either diphasic depressor-pressor or purely depressor effects depending chiefly on the nitrogen substituent. The secondary *1,2,3,4*-tetrahydroisoquinoline derivatives (type III) which have methoxy and ethoxy groups in the *6,7* positions were predominantly depressor in action. The tertiary *6*-ethoxy-*7*-methoxy dihydro- and tetrahydroisoquinoline derivatives were depressor while the other derivatives had mixed depressor-pressor effects. The quaternary dihydroisoquinolinium salts (type II) also had mixed depressor-pressor actions. In general, the substitution of a methyl group in the *1*-position had relatively little influence on the blood pressure response since similar qualitative effects were obtained with the compounds unsubstituted in the *1* position (2, 3).

TABLE 2

*Toxicological effects of 1-methyl-3,4-dihydro- and 1-methyl-1,2,3,4-tetrahydroisoquinoline derivatives on albino mice*

| NO. | COMPOUND TYPE | SUBSTITUENT GROUPS | MOLECULAR WEIGHT | LD 50 | SLOPE | LIMITS OF ERROR | PILOMOTOR EFFECTS | EXOPHTHALMOUS | SALIVATION | TREMORS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *mgm./kgm.* | | % | | | | |
| 734 | III | *6,7*-Dihydroxy | 215.6 | 417 | 15 | 92–108 | | Moderate | Moderate | |
| 732 | III | *6,7*-Dimethoxy | 243.6 | 189 | 16 | 94–106 | | | | Coarse |
| 731 | III | *6*-Ethoxy | 227.6 | 127 | 12 | 90–111 | | | | Coarse |
| 733 | III | *6*-Ethoxy-*7*-methoxy | 257.6 | 93 | 19 | 92–109 | | | | Intermittent |
| 740 | III | *2*-Methyl | 197.6 | 77 | 31 | 94–106 | | | | Moderate |
| 744 | IV | *2*-Methyl-*6,7*-dihydroxy | 229.6 | 169 | 16 | 89–112 | | Slight | | |
| 742 | IV | *2*-Methyl-*6,7*-dimethoxy | 257.6 | 26 | 12 | 93–108 | | Moderate | | |
| 741 | IV | *2*-Methyl-*6*-ethoxy | 241.6 | 55 | 19 | 94–107 | | | Frequent | Frequent spasms |
| 743 | IV | *2*-Methyl-*6*-ethoxy-*7*-methoxy | 271.6 | 11 | 40 | 97–103 | | | | Coarse |
| 730 | I | *6,7*-Dihydroxy | 213.6 | 207 | 22 | 93–107 | | | | |
| 728 | I | *6,7*-Dimethoxy | 241.6 | 108 | 23 | 94–108 | | | | Persistent, fine |
| 727 | I | *6*-Ethoxy | 225.6 | 80 | 37 | 96–104 | | | | Persistent, fine |
| 729 | I | *6*-Ethoxy-*7*-methoxy | 255.6 | 48 | 32 | 93–107 | | | Slight | Violent |
| 739 | II | *2*-Methyl-*6,7*-dihydroxy | 227.6 | 119 | 12 | 88–114 | | Slight | | Moderate |
| 736 | II | *2*-Methyl-*6,7*-dimethoxy | 255.6 | 56 | 15 | 93–108 | | Moderate | | |
| 735 | II | *2*-Methyl-*6*-ethoxy | 239.6 | 141 | 29 | 94–106 | | | | Occasional |
| 738 | II | *2*-Methyl-*6*-ethoxy-*7*-methoxy | 269.6 | 60 | 13 | 93–108 | Slight | Slight | | *Fine* |

Roman numerals refer to compounds the formulae of which are given in the introduction.

There was some evidence of tachyphylaxis among the dihydroxy pressor agents, paired doses following the series of graded doses showing decreased effects.

Likewise among the depressor-pressor substances paired doses were frequently purely depressor, the animal having become insensitive to the pressor phase.

Vagotomy and atropine usually had no influence on the response as shown by paired doses, indicating the absence of a parasympathetic component in the blood pressure response.

TABLE 3

*Relative effects of 1-methyl isoquinoline compounds on blood pressure, respiration and pulse*

| NO. | COMPOUND TYPE | SUBSTITUENT GROUPS | DOSE | | BLOOD PRESSURE | | | | PAIRED DOSE | EFFECT OF VAGOTOMY | EFFECT OF ATROPINE | EFFECT ON EPINEPHRINE | RESPIRATION | PULSE | TREMORS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Change | | Duration | | | | | | | | |
| | | | *mM / kgm.* | *mgm / kgm* | *mm. Hg* | | *minutes* | | | | | | | | |
| 734 | III | *6*,*7*-Dihydroxy | .004 | .86 | | p 41 | | 2 | 0 | 0 | 0 | S | 0 | 0 | 0 |
| | | | 032 | 6.88 | | p 85 | | 8 | | | | | | | |
| 732 | III | *6*,*7*-Dimethoxy | .008 | 1 95 | d 16 | | 2 | | 0 | 0 | 0 | 0 | I | 0 | 0 |
| | | | .064 | 15.20 | d 60 | | 15 | | | | | | | | |
| 731 | III | *6*-Ethoxy | .008 | 1.82 | d 28 | | 1 | | 0 | 0 | 0 | D | I | 0 | + |
| | | | 064 | 14 56 | d 110 | | 12 | | | | | | | | |
| 733 | III | *6*-Ethoxy-*7*-methoxy | .008 | 2.06 | d 30 | p 15 | 1 | 2 | d | 0 | 0 | D | I | 0 | +++ |
| | | | .032 | 8.24 | d 37 | | 15 | | | | | | | | |
| 740 | IV | *2*-Methyl | .008 | 1 58 | d 18 | p 12 | <1 | 2 | 0 | 0 | 0 | R | I | 0 | + |
| | | | .032 | 6.32 | d 12 | p 15 | <1 | 5 | | | | | | | |
| 744 | IV | *2*-Methyl-*6*,*7*-dihydroxy | 002 | .46 | | p 29 | | 2 | 0 | 0 | 0 | S | 0 | 0 | 0 |
| | | | 032 | 7.34 | | p 97 | | 15 | | | | | | | |
| 742 | IV | *2*-Methyl-*6*,*7*-dimethoxy | 008 | 2.06 | d 18 | p 15 | <1 | 3 | D | 0 | 0 | D | I | D | +++ |
| | | | .064 | 16.48 | d 42 | p 67 | <1 | 5 | | | | | | | |
| 741 | IV | *2*-Methyl-*6*-ethoxy | .008 | 1.93 | d 24 | p 21 | 1 | 10 | d | 0 | 0 | D | I | 0 | +++ |
| | | | .064 | 15.46 | d 115 | | 60 | | | | | | | | |
| 743 | IV | *2*-Methyl-*6*-ethoxy-*7*-methoxy | 008 | 2.17 | d 40 | | 2 | | 0 | 0 | 0 | D | I | 0 | +++ |
| | | | 032 | 8.68 | d 75 | | 10 | | | | | | | | |
| 730 | I | *6*,*7*-Dihydroxy | .008 | 1.71 | | p 13 | | 10 | D | 0 | 0 | S | 0 | D | 0 |
| | | | .032 | 6.84 | | p 24 | | 12 | | | | | | | |
| 728 | I | *6*,*7*-Dimethoxy | .008 | 1 93 | d 7 | p 15 | <1 | 5 | d | 0 | 0 | S | 0 | 0 | ++ |
| | | | 032 | 7.72 | d 52 | p 42 | 2 | 20 | | | | | | | |
| 727 | I | *6*-Ethoxy | 008 | 1 80 | d 7 | p 12 | <1 | 5 | d | 0 | 0 | S | I | 0 | + |
| | | | 032 | 7.22 | d 35 | p 15 | 1 | 15 | | | | | | | |
| 729 | I | *6*-Ethoxy-*7*-methoxy | .008 | 2.05 | d 16 | | 1 | | 0 | 0 | 0 | S | I | D | ++ |
| | | | .064 | 16 36 | d 90 | | 20 | | | | | | | | |
| 739 | II | *2*-Methyl-*6*,*7*-dihydroxy | .008 | 1.82 | | p 19 | | 1 | D | 0 | 0 | S | I | 0 | + |
| | | | 032 | 6.32 | d 45 | p 35 | <1 | 5 | | | | | | | |
| 736 | II | *2*-Methyl-*6*,*7*-dimethoxy | .008 | 2.04 | d 15 | p 15 | <1 | 4 | d | 0 | 0 | S | I | 0 | 0 |
| | | | .064 | 16.36 | d 50 | | 15 | | | | | | | | |
| 735 | II | *2*-Methyl-*5*-ethoxy | 008 | 1.92 | d 9 | p 10 | <1 | 1 | 0 | 0 | 0 | S | I | D | 0 |
| | | | .064 | 15.32 | d 34 | p 37 | 3 | 10 | | | | | | | |
| 738 | II | *2*-Methyl-*6*-ethoxy-*7*-methoxy | .008 | 2.16 | d 6 | p 25 | <1 | 4 | 0 | 0 | 0 | S | I | 0 | + |
| | | | .032 | 8 64 | d 37 | p 36 | 1 | 4 | | | | | | | |

*d*, depressor; *p*, pressor; *D*, decrease; *I*, increase; *0*, no change, *S*, sensitized, +, moderate tremors; ++, marked tremors, +++, strychnine-like tremors, *R*, reversal

Each compound was tested on two dogs

Roman numerals refer to compounds the formulae of which are given in the introduction.

The epinephrine response was usually increased by the dihydroisoquinoline compounds regardless of the substituents in the *6*,*7* positions. It was also increased by the dihydroxy pressor agents among the tetrahydroisoquinoline derivatives while it was usually inhibited by the methoxy and ethoxy tetrahydroisoquinoline derivatives. The epinephrine response was actually reversed by large doses of the unsubstituted *1,2*-dimethyl-*1,2,3,4*-tetrahydroisoquinoline. The analogous compound, unsubstituted in the *1* position, was likewise adrenolytic (2).

There was usually an initial brief respiratory stimulation with these isoquinoline derivatives. The pulse rate was usually unchanged; only an occasional compound decreased the rate. The majority of the compounds produced tremors in the anesthetized dog. Four of the tetrahydro derivatives, with ethoxy and *methoxy substituents, produced violent strychnine-like convulsions of long* duration.

TABLE 4

*Relative effects of 1-methyl isoquinoline compounds on tone of isolated smooth muscle*

| NO | COMPOUND TYPE | SUBSTITUENT GROUPS | CONCENTRATION IN BATH | RABBIT INTESTINE | RABBIT UTERUS | GUINEA PIG UTERUS | NUMBER OF TESTS |
|---|---|---|---|---|---|---|---|
| | | | *milli-molar* | | | | |
| 734 | III | *6,7*-Dihydroxy | 0.1 –0.4 | (+ –) | + | + | 3 |
| 732 | III | *6,7*-Dimethoxy | 0.1 –0.2 | – | + | + | 3 |
| 731 | III | *6*-Ethoxy | 0.1 –0.2 | – – | + + | + + | 4 |
| 733 | III | *6*-Ethoxy-*7*-methoxy | 0.1 –0.2 | – – | + | + | 3 |
| 740 | IV | *2*-Methyl | 0.1 –0.2 | – – | + + | + + | 6 |
| 744 | IV | *2*-Methyl-*6,7*-dihydroxy | 0.1 –0.2 | (+ –) | + + | + + | 4 |
| 742 | IV | *2*-Methyl-*6,7*-dimethoxy | 0.1 –0.4 | – | + | + + | 4 |
| 741 | IV | *2*-Methyl-*6*-ethoxy | 0.1 –0.2 | – | + | + | 3 |
| 743 | IV | *2*-Methyl-*6*-ethoxy-*7*-methoxy | 0.1 –0.4 | – | + + | + + | 4 |
| 730 | I | *6,7*-Dihydroxy | 0.1 –0.4 | (+ –) | + | + | 4 |
| 728 | I | *6,7*-Dimethoxy | 0.1 –0.2 | – | + | + + | 3 |
| 727 | I | *6*-Ethoxy | 0.05–0.2 | – | + + | + + | 3 |
| 729 | I | *6*-Ethoxy-*7*-methoxy | 0.05–0.2 | – | + | + + | 3 |
| 739 | II | *2*-Methyl-*6,7*-dihydroxy | 0.05–0.2 | (+ –) | + | + | 5 |
| 736 | II | *2*-Methyl-*6,7*-dimethoxy | 0.1 –0.2 | – | + | + + | 3 |
| 735 | II | *2*-Methyl-*6*-ethoxy | 0.1 –0.2 | – | + | + | 3 |
| 738 | II | *2*-Methyl-*6*-ethoxy-*7*-methoxy | 0.1 –0.2 | – | + + | + + | 7 |

+, moderate stimulation; + +, marked stimulation; –, moderate inhibition; – –, marked inhibition; (+ –), mixed stimulation and inhibition.

Roman numerals refer to compounds the formulae of which are given in the introduction.

*IV. Smooth muscle effects.* The data on isolated smooth muscle, obtained by previously described methods (13) are summarized in table 4. The dihydroxy derivatives usually had mixed stimulating and depressing effects on intestine while the remaining derivatives usually depressed. All of the compounds stimulated both rabbit and guinea pig uteri. These reactions were consistent with those for the corresponding isoquinoline derivatives unsubstituted in the *1* position (2, 3).

## SUMMARY

A series of *1*-methyl-*3,4*-dihydro- and *1*-methyl-*1,2,3,4*-tetrahydroisoquinoline derivatives substituted in the *6* and *6,7* positions with hydroxy, methoxy

and ethoxy groups and having in the 2 position hydrogen or methyl, were studied for their chemical and relative pharmacological properties.

The presence of hydroxy groups at positions *6* and *7* seemed to decrease toxicity. Usually the quaternary salts were more toxic than the secondary and tertiary amines.

The *1*-methyl substituent had relatively little influence on the qualitative blood pressure responses; the effects of the present series were similar in most respects with those of the *1*-unsubstituted analogs.

Tachyphylaxis of the pressor responses was occasionally noted.

Vagotomy and atropine did not influence the blood pressure responses.

Respiration was consistently stimulated. The pulse rate was usually unchanged.

Epinephrine was potentiated by the dihydroisoquinoline compounds and by the dihydroxy tetrahydroisoquinoline derivatives; it was inhibited by the other tetrahydro derivatives.

Tremors were observed with the majority of the compounds. Four of the tetrahydroisoquinoline derivatives produced strychnine-like convulsions.

The isolated intestine was either stimulated or relaxed or both by the dihydroxy derivatives but depressed by all other compounds. The uteri were consistently stimulated.

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# THE CHRONOLOGICAL RELATIONSHIP OF THE BLOOD PRESSURE AND SERUM POTASSIUM EFFECTS OF EPINEPHRINE[1]

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Considerable interest in potassium salts has been evident in the clinical literature of recent years. Potassium salts have been recommended in the treatment of urticaria by Rusk and Kenamore (1); in hay fever, urticaria, eczema, nasal polyposis, chronic allergic sinusitis and migraine by Bloom (2). Bloom also recommends potassium iodide for asthma although he states that potassium chloride has no effect. Harsh and Donovan (3) have failed to confirm Bloom's results and have shown that blood potassium levels are unchanged by the oral doses used.

These purely clinical studies, with the exception of the last, had their origin, in part, in the theory of Camp and Higgins (4) which may be stated as follows: epinephrine causes an increase in serum potassium, injected potassium salts cause a rise in blood pressure, therefore the effect of epinephrine on the blood pressure is due to the increase of blood potassium. The previous work on which this theory was mainly based was that of Mathison (5) who showed that injected potassium salts cause an epinephrine-like rise of blood pressure, and that of Schwarz (6) who showed that injected epinephrine caused an increase in blood potassium. The authors of this theory ignored Mathison's further conclusions that potassium caused contraction of plain muscles, such as the oesophagus and uterus of the cat, which are relaxed by epinephrine; and also Schwarz' conclusion that the "potassium increase and blood pressure rise can be brought into no determined numerical relation."

Several previously published papers contained evidence tending to refute a potassium theory of epinephrine action. Thus Hazard and Wurmser (7) showed that magnesium chloride prevented the rise in blood pressure caused by injected potassium salts but did not interfere with the blood pressure rise caused by epinephrine. Bacq and Rosenblueth (8) found that in an adrenalectomized cat potassium salts caused a contraction of the uterus while epinephrine caused relaxation. Zwemer and Truskowski (9) reported that potassium salts injected into animals caused all the symptoms of adrenal insufficiency, simulating Addison's disease. The fact was demonstrated by Howell and Duke (10), Martin (11), and others, that potassium salts affect the heart like vagus stimulation.

About the time of the publication of the theory of Camp and Higgins, Marenzi and Gershman (12) showed that when the hepatic veins were ligated a rise of

[1] This work was presented as partial fulfillment of the requirements for the Ph.D. degree in Pharmacology at the University of Michigan.

plasma potassium no longer resulted from epinephrine injection. D'Silva (13) confirmed this and added the observation that perfused liver releases potassium when epinephrine is added to the perfusion fluid. Subsequently (14) he showed that after successive 0.1 mgm. doses of epinephrine, three minutes apart, the mobilization of potassium was feeble after the seventh injection. Larson and Brewer (15) concluded that "there is no causal relationship between the rise in serum potassium and the rise in blood pressure resulting from the injection of adrenalin" on the basis of the practical disappearance of the potassium-mobilizing effect of adrenalin after hepatectomy, while the blood pressure effect remained unimpaired. Knoeffel and Alles (16) also took exception to Camp's theory on the basis of difference in pharmacological action of epinephrine and potassium salts similar to those already quoted.

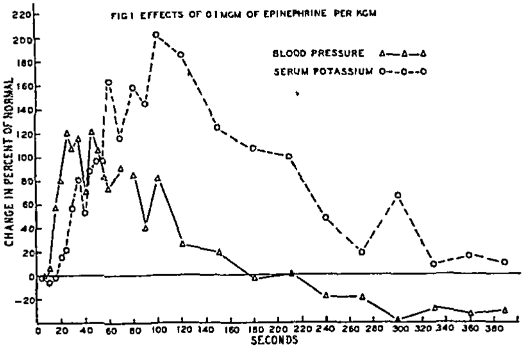

FIG. 1. EFFECTS OF 0.1 MGM. PER KGM EPINEPHRINE ON SERUM POTASSIUM AND BLOOD PRESSURE

Though the potassium theory of epinephrine action has been disproved, the time relations between these two actions of epinephrine have not been studied. The object of the present work is to demonstrate this relation.

PROCEDURE. The experiments were carried out on cats under nembutal anesthesia. The carotid blood pressure was recorded by a mercury manometer. Blood samples were collected from a femoral artery through a special cannula made from an 18 gauge hypodermic needle. This cannula had the hub of the needle soldered in the side and the arterial flow continued through the lumen of the needle, which was placed in the lumen of the artery and tied in at each end. Injections of epinephrine were made into a small branch of the opposite femoral vein. *Blood samples were taken immediately before the injection to serve* as a normal After the injection the samples were taken as rapidly as possible. Immediately after coagulation they were centrifuged and the serum separated from the cells.

The serum potassium was determined by the method described by Hoffman (17) using an Evelyn photoelectric colorimeter and a 600 mμ. filter. The calibration curve was made using known amounts of potassium precipitated in the same manner as the serum potassium. All determinations were made in duplicate.

Results. The results in the graphs are expressed as changes from the normal in percentage of the normal value. The graphs plotted from the average of results from sixteen doses of 0.1 mgm. (in 10 cats) (fig. 1) and from sixteen doses of 0.05 mgm. (in 8 cats) (fig. 2) of epinephrine per kgm. of body weight show:

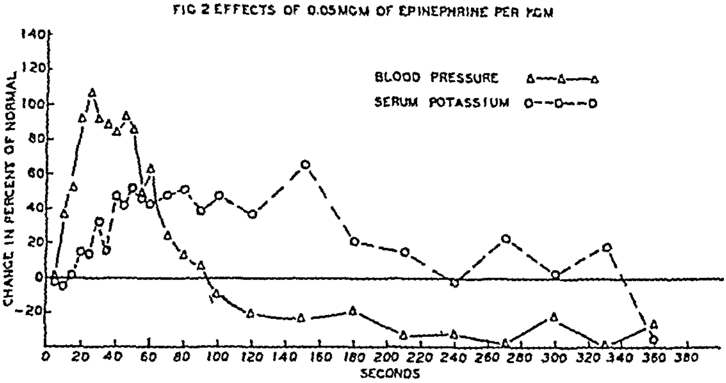

Fig. 2. Effects of 0.05 mgm. per kgm. Epinephrine on Serum Potassium and Blood Pressure

TABLE 1

*Time relations of blood pressure and serum potassium after epinephrine*

| DOSE | TIME (SECONDS) | | | | | |
|---|---|---|---|---|---|---|
| | Beginning of rise | | Height of rise | | Return to normal | |
| | B. P. | K | B. P. | K | B. P. | K |
| mgm. 0.1 | 10 | 20 | 25 | 100 | 180 | 320 |
| Difference | 10 | | 75 | | 140 | |
| 0.05 | 10 | 20 | 25 | 45 | 120 | 240 |
| Difference | 10 | | 20 | | 120 | |

The blood pressure rose within ten seconds after the injection, while the increase in serum potassium began, within twenty seconds. The potassium increase was relatively greater than the blood pressure increase in the animals receiving the 0.1 mgm. dose, relatively less than the blood pressure increase in the animals receiving the 0.05 mgm. dose. The peak of the blood pressure rise was past and the return to normal began before the peak of the potassium increase was reached, and was close to the beginning of the potassium rise. As shown by table 1, the effect on serum potassium was slower throughout than the blood pressure effect.

Discussion. All of these findings are definitely against the idea that the blood pressure effect of epinephrine is due to the increase of blood potassium. D'Silva (13) and Marenzi and Gershman (12) showed that most of the potassium liberated by epinephrine came from the liver. This would account for the delay in the appearance of the potassium in the blood of the femoral artery and point further toward the conclusion that the potassium-mobilizing effect of epinephrine is independent of the blood pressure effect. If the increase in serum potassium caused by release of potassium from the liver were the cause of the vasoconstriction responsible for the increase of blood pressure, the serum potassium in the blood of the femoral artery should rise before the blood pressure rises, since blood from the heart would reach the femoral artery before it reaches the arterioles and capillaries of the leg and at about the same time that it reaches the visceral arterioles and capillaries. Since Briggs, Koechig, Doisy and Weber (18) Harrop and Benedict (19) and Kerr (20) showed that insulin caused a decrease in blood potassium and since D'Silva (21) showed that the fall of blood potassium following the rise provoked by epinephrine was abolished by pancreatectomy, it is more likely that the potassium changes occurring after epinephrine are related to the hyperglycemia rather than the hypertension induced by epinephrine, as suggested by Harrop and Benedict (19) in the case of the serum potassium change induced by insulin. Such relations must be left to subsequent study before any conclusion is reached.

## CONCLUSIONS

1. The serum potassium increase caused by injected epinephrine is slower and more prolonged than the blood pressure increase.
2. The serum potassium increase caused by 0.1 mgm. per kgm. of epinephrine in the cat is relatively greater than the blood pressure effect. That caused by 0.05 mgm. per kgm. is relatively less than the blood pressure effect.
3. The serum potassium increase after the injection of epinephrine can not be the cause of the blood pressure increase.

*Acknowledgement.* Appreciation is expressed to Doctor Ralph G. Smith for his criticism and advice.

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# A DEVICE FOR THE CONTINUOUS RECORDING OF HEART RATE: ITS APPLICATION TO A STUDY OF THE CIRCULATORY EFFECTS OF EPINEPHRINE AND CARBAMINOYL CHOLINE

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In the course of a study on the influence of the vagus upon changes in heart rate following the administration of various drugs, it became necessary to record the frequency of the heart beat continuously over extended periods of time. This communication presents findings on the effect of epinephrine and carbaminoyl choline on the heart rate of rabbits and cats as recorded by a new device for the continuous recording of the heart rate.

METHOD. Present methods for determining the heart rate consist in recording the individual heart beats either as *mechanical* or *electrical* impulses. The recording of mechanical impulses requires a minimum of inertia of the recording instrument and a maximum of speed of the kymograph. Ordinary kymographs designed for smoked paper or ink recording are too slow, particularly for experiments on small animals with heart frequencies of more than 150 per minute. The disadvantages encountered in mechanical arrangements are avoided in methods such as the electrocardiograph, which record the electric impulses of the heart beat by photographic means. However, the electrocardiogram as a means for determining the heart rate has certain disadvantages. In experiments in which simultaneously with the heart rate, changes in volume and pressure are recorded on a smoked paper kymograph, it is inconvenient to analyze the results on two separate records which are not synchronized. Furthermore, the cost of photographic paper prohibits the use of the electrocardiogram for continuous recording of more than a few minutes, and finally, the electrocardiogram shares with all photographic recordings the disadvantage that results cannot be read while the experiment is still in progress.

For these reasons, we have developed a method which permits recording of the heart rate continuously on an ordinary smoked paper or ink kymograph simultaneously with other recordings. Our method is based on amplifying the heart potentials and using the amplified impulses to actuate a combined mechanical counting and recording device. The heart potentials picked up by needle electrodes from the animal are amplified by a portable A.C.-operated amplifier. The amplified impulses are counted by a mechanical counter which consists of a step motor advancing an equal increment for each impulse. The total rotation for a given period (10 seconds) is transmitted by the recorder to a writing point on the kymograph as a linear vertical displacement. The recorder maintains a permanent calibration, and the distance of the recorded curve from its base line is directly proportional to the heart rate.

This method of averaging and recording the heart rate compares quite favorably with that employing the electronic integrating circuits which, for slow rates, require heavy damping. Furthermore, electronic integrators necessitate a separate recorder and frequent calibration, since the rate is measured indirectly in terms of current or voltage.

For reasons of flexibility, the instrument is divided into two separate units, the amplifier (fig. 1) and the counter (fig. 2).

*Amplifier.* The amplifier is a straight-forward condenser coupled type, operated entirely from the 110 volt A.C. line. The amplifier chassis also carries a two inch cathode ray tube with a variable sweep for observing the electrocardiogram, and a trigger circuit for actuating the relay for the counter and recording mechanism.

As shown in the wiring diagram (fig. 1), an interference cancelling circuit has been incorporated between the first and second stages of the amplifier. Its purpose is to inject a 60 cycle voltage of variable phase and amplitude, and thereby cancel any extraneous 60 cycle interference that may be picked up due to inadequate shielding of the animal.

The trigger circuit is the conventional two stage feedback amplifier biased to cutoff. Since the circuit will trip only on a negative pulse, a phase inverter tube has been provided which, together with a single pole double throw switch, makes it possible to present the grid of the trigger tube with a pulse always negative with respect to its cathode. The uniform current pulse delivered by the above circuit actuates a relay which in turn makes and breaks the low voltage circuit of the counter.

*Counter mechanism.* The counter consists of two electromagnetic step motors (fig. 2, *1* and *2*) which are alternately connected to the output relay of the amplifier for a period of 10 seconds. Since the step motors are started from zero, the degree of rotation is always proportional to the number of impulses received.

To record the number of impulses counted, each motor has attached to its shaft a commutator (*3* and *4*) with two insulated copper segments. Corresponding segments from each disc are connected to the forward and reverse fields of the recording motor (*6*). Since the power is conveyed to the copper segments by means of the sliding finger (*5*) coupled mechanically to the recording motor, the motor always rotates towards the same insulated section between the two segments. The motion of the motor is transmitted simultaneously to a writing point on a recording drum by means of a cable wound around a pulley (*7*). The shaft in the step motor (*1*) is hollow and the shaft of the recording motor passes through it to the sliding contact. To maintain the proper sequence of events, a timing disc (*8*) is provided, driven by a 6 R.P.M. synchronous motor.

For the purpose of analyzing the sequence of events let us assume that impulses are coming through the amplifier, with step motor no. *1* just completing its 10 second counting period and motor no. *2* being set at zero. The first contact (*A*) on the timing disc operates a ratchet relay (*9*) which disconnects step motor no. *1* and simultaneously connects step motor no. *2* to the output relay for counting. The second contact (*B*) completes the circuit of the recording motor up to the segmented disc of step motor no. *1*. The recording motor then revolves in the proper direction until it comes to rest upon the insulated section. The speed of the recording motor is such that it reaches the insulated section within the allotted time of 8 seconds. The third contact (*C*) on the timing disc separates the electromagnetic ratchet release on motor no. *1* resetting it to zero. Here the time involved is well under 2 seconds, so that the entire operation of recording and resetting is within the 10 second counting interval. Consequently when the first contact (*A*) on the timing disc again operates the ratchet switch (*9*) at the 10 second period, motor no. *1* immediately takes up the count, while motor no. *2* stops to be recorded and reset.

Figure 3 shows the type of record obtained with the rate of impulses being changed from 20 to 19 and 30 per 10 seconds. A difference of 1 impulse per 10 seconds is clearly visible (3.5 mm. on the original record). The frequency per minute is obtained by multiplying the number of impulses by the reciprocal of the counting period in minutes, i.e., times 6, in case of a counting period of 10 seconds. The value thus obtained represents the actual heart rate provided the same frequency is maintained for at least one counting period.

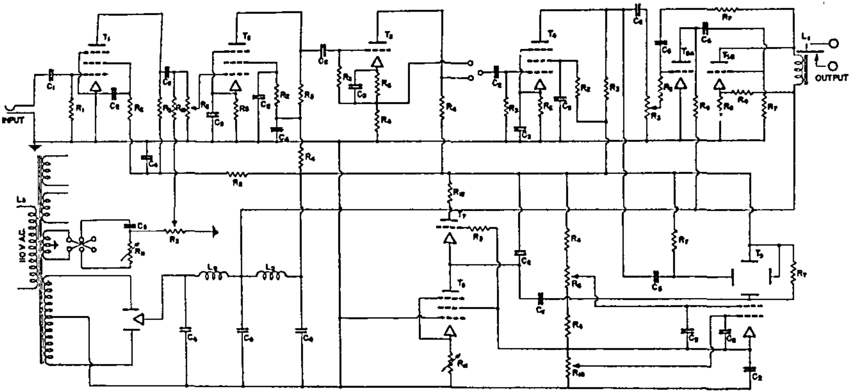

INPUT
OUTPUT
110 V AC
$T_1$
$T_2$
$T_3$
$T_4$
$T_{5a}$
$T_{5b}$
$T_6$
$T_7$
$T_9$
$L_1$
$L_2$
$L_3$

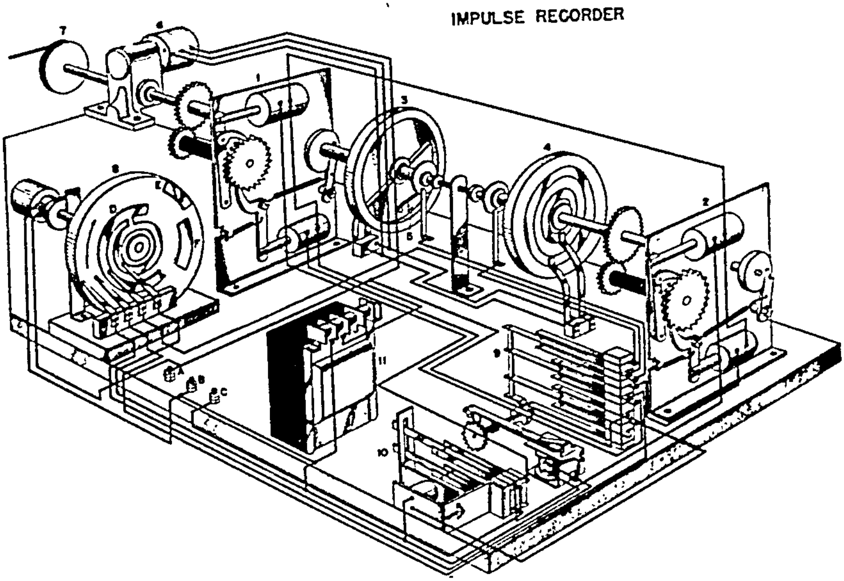
FIG. 1. SCHEMATIC DIAGRAM OF AMPLIFIER

$C_1 = 0.01$
$C_2 = 0.1$
$C_3 = 40.0$
$C_4 = 8.0$
$C_5 = 0.05$
$C_6 = 0.5$
$C_7 = 16.0$

$R_1 =$ 10 megs.
$R_2 =$ 2 megs.
$R_3 =$ 0.5 meg.
$R_4 =$ 0.1 meg.
$R_5 =$ 1250 ohms
$R_6 =$ 10,000 ohms.
$R_7 =$ 1.0 meg.
$R_8 =$ 30,000 ohms.

$R_9 =$ 15,000 ohms
$R_{10} =$ 20,000 ohms
$R_{11} =$ 50,000 ohms
$R_{12} =$ 1,000 ohms
$R_{13} =$ 5 megs.
$L_1 =$ 10,000 ohm coil relay
$L_2 =$ 30 Henries
$L_3 =$ 730 v ct. transformer

$T_1, T_2, T_4, T_8 =$ 6SJ7
$T_3 =$ 6C5
$T_5 =$ 6C8G
$T_6 =$ 83-V
$T_7 =$ 884
$T_9 =$ 902


IMPULSE RECORDER
1
2
3
4
5
6
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8
9
10
11
A
B
C
D
E
F
G
H

FIG. 2. IMPULSE COUNTER AND RECORDER

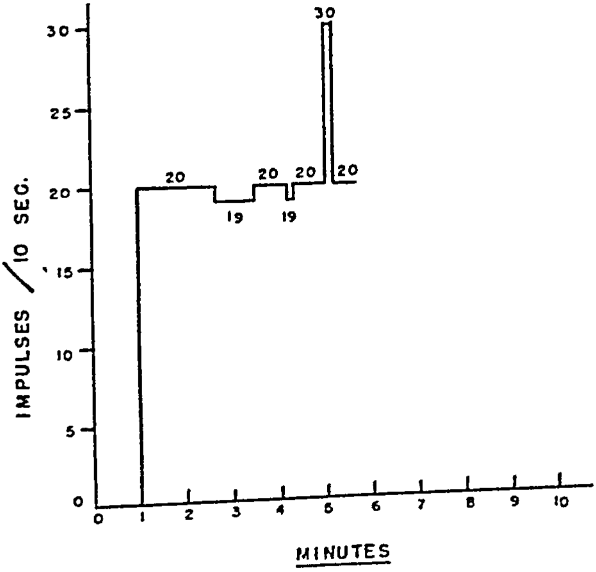

FIG. 3. TYPE OF RECORD OBTAINED FROM THE IMPULSE COUNTER AND RECORDER

The counting period may be varied, a longer period being used when the heart beat is irregular, in order to obtain a representative average, and a shorter period being preferred where short lasting changes of the heart rate are expected. The error is equivalent to the ratio of one beat to the total number of beats for a single counting period; its magnitude, therefore, increases with the shortening of the counting period. This difference of one beat occurs when an impulse arrives at the moment a counting period is being terminated. In experiments on cats and rabbits (heart rates of 200–400 per minute) a 10 second counting period gave us the most satisfactory results with an error of 2 to 5%.

The counter is adapted to record frequencies up to 450 per minute. It is obvious that the counter does not yield any information about the character of the heart beat nor its

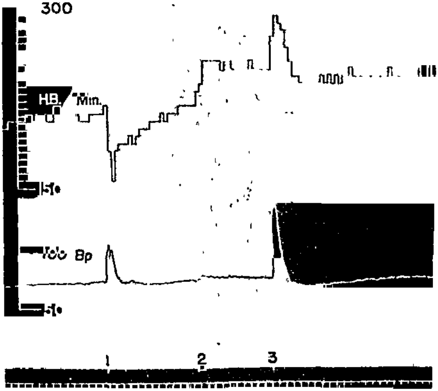

FIG. 4. EFFECT OF EPINEPHRINE ON THE HEART RATE

Cat—3.2 kgm. body weight, 0.8 gram per kgm. urethane and 0.06 gram per kgm. chloralose.

*H.R.* = Heart rate, beats per minute; *B.P.* = carotid blood pressure, mm. Hg. Time in 30 seconds. Intravenous injections at *1* and *3*–40 micrograms of epinephrine; at *2*—0.25 mgm. atropine sulfate.

wave shape, but merely records the peak voltage of the Q.R.S. complex as a single impulse. On the other hand, as a device for continuous recording of mechanical impulses, it holds a diversified field of usage, already it has been satisfactorily used as a drop recorder.

RESULTS. Figure 4 demonstrates the changes of the heart rate of an anesthetized cat caused by epinephrine. The injection of 40 micrograms of epinephrine is followed by a marked decrease of the heart rate from 210 to 156 per minute, due to activation of pressoreceptors in the carotids and aorta, with consequent stimulation of the vagus. Within 5 minutes the heart rate returns to its original level. Atropine (0.25 mgm.) increases the heart rate to 258 beats per minute. A subsequent injection of epinephrine (40 micrograms) now causes a sharp rise in the heart rate, together with a much more marked response of the blood pressure.

Figure 5 demonstrates the changes of the heart rate of a rabbit following the intravenous injection of carbaminoyl choline (Lentin). Graded doses of this compound (1, 2 and 3 micrograms) produce graded responses in the heart rate, decreases of a maximum of 24 (1 microgram), 48 (2 micrograms) and 60 beats per minute (3 micrograms) being recorded in this experiment. The simultaneous changes in the blood pressure are only slight and are of shorter duration than the corresponding changes in the heart rate. The administration of atropine raises the frequency to 290 beats per minute and abolishes the effects of subsequent injections of carbaminoyl choline upon both the heart rate and the blood pressure.

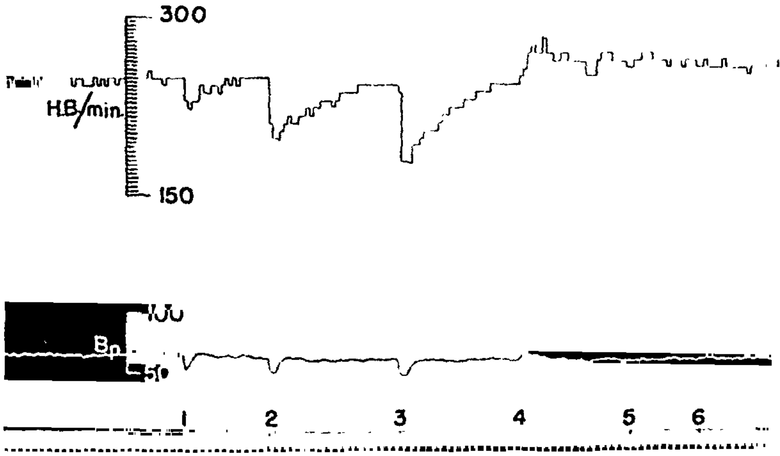

FIG. 5. EFFECT OF CARBAMINOYL CHOLINE ON THE HEART RATE

Rabbit: 3.0 kgm. body weight; 0.8 gram per kgm. urethane and 0.06 gram per kgm. chloralose.

*H. R.* = Heart rate, beats per minute.

*B. P.* = Carotid blood pressure, mm. Hg. Time in 30 seconds. Intravenous injections of carbaminoyl choline at *1*-1 microgram; at *2*-2 micrograms; at *3*-3 micrograms; at *5*-2 micrograms; at *6*-3 micrograms. At *4*-0.25 mgm. of atropine sulfate.

The effects obtained with epinephrine and carbaminoyl choline demonstrate that changes in the heart rate due to vagal stimulation are of considerably longer duration than the concomitant changes in the blood pressure. Whereas the blood pressure returns to its original level within 90 seconds, about 5 minutes are required for the heart to attain its previous frequency. In the atropinized animal, on the other hand, the period of acceleration of the heart rate following epinephrine coincides with the duration of the effect upon the blood pressure.

### SUMMARY

1. A method is described which permits the continuous recording of the heart rate on an ordinary smoked paper or ink kymograph.

2. Epinephrine (40 micrograms) and carbaminoyl choline (1–10 micrograms) decrease the heart rate. The depression of the heart rate due to vagal stimulation lasts considerably longer than the concomitant changes in the blood pressure.

3. In the atropinized cat, the increase in the heart rate following epinephrine is of the same duration as the increase in blood pressure.

# COMPARATIVE STUDIES ON THE TOXIC EFFECTS OF DIGITOXIN AND OUABAIN IN CATS

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Discrepancies between the potency of digitalis preparations or of individual glucosides, expressed by biological units, and their efficacy in therapeutics, have repeatedly been pointed out by numerous investigators. Bioassays in animals, based upon toxic effects produced by parenteral injection, disregard differences in absorption, tolerance, cumulation and other properties which are of utmost importance in clinical application. Although a bioassay is indispensable at present to ensure uniform strength of a digitalis preparation, the potency established in animals is applicable to man within certain limits only. Two preparations of different composition possessing the same potency in one animal species do not necessarily produce identical effects in man, and further evaluation of the merit of a given preparation rests upon clinical observation. Apart from consideration of differences in chemical structure and *physico-chemical* properties, which have been the object of numerous investigations on stability, potency, absorption and elimination of digitalis and its allies, there remains the question whether there are *qualitative differences in the specific cardiac effect* among different glucosides. That such differences in the pharmacologic effect upon the heart exist, is the assumption of many clinicians (1). But, according to Cattell and Gold (2), "Conclusive experimental evidence bearing on this problem is wanting." Gold and coworkers (3, 4), comparing 6 widely different preparations including tincture of digitalis, digitoxin and ouabain in unanesthetized cats, were unable to find qualitative differences in the cardiac action as measured by the electro-cardiogram. A comparison of ouabain and digitoxin in their effect upon the isolated papillary muscle of the cat demonstrated no qualitative difference between the two glucosides (2). However, Moe and Visscher (5), analyzing the cardiac action of three glucosides of *Digitalis Lanata* in heart-lung preparations of dogs, obtained evidence of striking differences in the increase of mechanical efficiency of the heart as well as in the occurrence of cardiac irregularities. Furthermore, significant differences between digitoxin and digitoxigenin were observed (6) in their effects upon the conduction time, rate of the sinus rhythm and the occurrence of ectopic beats in cats anesthetized with ether.

In view of these conflicting results a comparative study on the cardiac action of two purified glucosides, digitoxin and ouabain, was undertaken in anesthetized cats. The data presented in this paper are intended as a contribution toward an analysis of qualitative differences between cardiac glucosides.

METHODS. The experiments were carried out on cats (total number 73, 50 males and 23 females) varying in body weight from 2.05 to 4.0 kgm. The animals were anesthetized with urethane and chloralose or with sodium pentobarbital. Urethane (1 gram per kgm. body weight) was given subcutaneously in a 25% aqueous solution 2 hours before the intravenous injection of 40 mgm. per kgm. of chloralose. This combination produced a satisfactory and apparently constant anesthesia, the corneal and tendon reflexes being present throughout the experiment. With sodium pentobarbital it was not possible to obtain uniform anesthesia in all animals by the administration of a single predetermined dose correlated to the weight of the animal. The dose of pentobarbital was 30 mgm per kgm. intraperitoneally, supplemented as required with varying additional small amounts given intravenously. The total amount of sodium pentobarbital required for the desired depth of anesthesia varied from 40 to 60 mgm. per kgm.; no addition injections were given during the infusion of the glucoside.

The animals were placed on an operating table heated to prevent a drop in body temperature. The infusion of the glucoside into a jugular or femoral vein was given by a pump driven by a constant speed motor.[1] In all experiments the injected volume was 0.9 cc. per minute. The concentration of the solution was adjusted to the weight of the animal and calculated to produce death in about 45 minutes. Alcoholic solutions of Digitoxin cryst. (Merck) and Ouabain (Merck), each of 0.1% strength, were diluted with saline, concentrations ranging from 0.0071% to 0 0137% of digitoxin and from 0.0019% to 0.0026% of ouabain being used.

The heart rate was measured and recorded continuously in all experiments by the heart rate recorder described by Kniazuk and Unna (7). Blood pressure in the carotid artery was recorded simultaneously by means of a mercury manometer.

The electrocardiogram, visualized on the screen of the oscillograph of the heart beat recorder, was observed throughout the experiments, and photographic records were taken at frequent intervals whenever required.

The infusion was commenced approximately 2 hours after the anesthetic had been given, when the anesthesia had reached a satisfactory constancy and only when the heart rate was constant and regular. Eight experiments (7 with sodium pentobarbital, 1 with chloralose anesthesia) were discarded because the heart rate of the animals was irregular. The infusion was carried out until cessation of the heart beat occurred as indicated in the majority of the experiments by an abrupt fall in blood pressure and verified by immediate opening of the thorax and inspection of the heart. The experiments with digitoxin and ouabain were alternated.

RESULTS. *Infusion of digitoxin.* Two series of experiments with digitoxin were carried out, one with sodium pentobarbital anesthesia, the other with urethane-chloralose anesthesia. The data obtained in the two series, involving 11 and 10 cats respectively, are summarized in tables 1 and 2.

Figure 1 presents the record of a typical experiment. The decrease in heart rate (sinus rhythm) was almost negligible during the infusion of the first third of the fatal dose. Thereafter a gradual decrease amounting to 22.5% of the original rate took place. In the majority of the experiments, the heart rate was slowest when the first ectopic beat was noticed. In a few experiments the slowest sinus rate was observed 1 or 2 minutes before the occurrence of ectopic beats, the heart beat, still in regular sinus rhythm, being accelerated by 6 to 18 per minute during this brief interval. The slowing of the heart rate was very variable in individual experiments (from 0-51%, see tables 1 and 2); in some, no significant decrease was observed at all. Apparently, the frequency of the heart beat at the beginning of the infusion bore no relation to the degree of slowing produced by the infusion of digitoxin. The wide variation in the decrease of the sinus rate was the same with either type of anesthesia.

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[1] The infusion pump was constructed by Mr. M. Kniazuk at our laboratory.

TABLE 1

*Infusion of digitoxin in cats anesthetized with sodium pentobarbital*

| NO. | WEIGHT OF ANIMAL | DURATION OF INFUSION | RATE OF INFUSION | SLOWEST SINUS RATE | | DECREASE IN SINUS RATE | FATAL DOSE | CARDIAC IRREGULARITY DOSE |
|---|---|---|---|---|---|---|---|---|
| | | | | Before infusion | During infusion | | | |
| | *kgm.* | *min.* | *mgm./min./kgm.* | | | *per cent* | *mgm./kg.* | *per cent of fatal dose* |
| 9 | 2.7 | 56 | 0.0075 | 212 | 144 | 32 | 0.421 | 73.7 |
| 10 | 2.2 | 39.3 | 0.0074 | 202 | 190 | 5.9 | 0.291 | 73.0 |
| 11 | 3.1 | 54.8 | 0.0071 | 272 | 258 | 5.1 | 0.387 | 71.0 |
| 17 | 3.2 | 59 | 0.0102 | 270 | 200 | 25.9 | 0.600 | 67.5 |
| 18 | 3.6 | 46 | 0.0113 | 210 | 186 | 11.4 | 0.521 | 72.5 |
| 19 | 2.5 | 54 | 0.0096 | 213 | 213 | 0 | 0.516 | 88.0 |
| 20 | 2.6 | 48 | 0.0090 | 194 | 156 | 19.5 | 0.433 | 71.0 |
| 21 | 2.5 | 64 | 0.0096 | 236 | 188 | 20.5 | 0.612 | 73.5 |
| 22 | 3.0 | 80 | 0.0103 | 184 | 134 | 27.2 | 0.820 | 71.0 |
| 61 | 2.67 | 43 | 0.0116 | 174 | 158 | 9.2 | 0.498 | 80.2 |
| 62 | 2.47 | 38.2 | 0.0125 | 186 | 144 | 22.5 | 0.478 | 78.6 |
| Mean | | | | | | | 0.507 | 74.5 |
| Standard error | | | | | | | 0.042 | ±1.7 |

TABLE 2

*Infusion of digitoxin in cats anesthetized with urethane-chloralose*

| NO. | WEIGHT OF ANIMAL | DURATION OF INFUSION | RATE OF INFUSION | SLOWEST SINUS RATE | | DECREASE IN SINUS RATE | FATAL DOSE | CARDIAC IRREGULARITY DOSE |
|---|---|---|---|---|---|---|---|---|
| | | | | Before infusion | During infusion | | | |
| | *kgm.* | *min.* | *mgm./min./kgm.* | | | *per cent* | *mgm./kgm.* | *per cent of fatal dose* |
| 5 | 2.5 | 37.4 | 0.0096 | 208 | 193 | 7.2 | 0.360 | 81.0 |
| 6 | 3 0 | 44.4 | 0.0105 | 190 | 176 | 7.3 | 0.467 | 72.1 |
| 23 | 3.35 | 42 | 0.0083 | 226 | 186 | 17.6 | 0.350 | 76.2 |
| 24 | 3.7 | 62.4 | 0.0074 | 188 | 168 | 10.5 | 0.459 | 80.2 |
| 25 | 2.9 | 50 | 0.0083 | 154 | 118 | 23.4 | 0.414 | 72.0 |
| 26 | 4.0 | 54 | 0.0082 | 270 | 174 | 35.5 | 0.415 | 77.7 |
| 27 | 2 95 | 71 | 0.0077 | 140 | 93 | 33.5 | 0.549 | 78.9 |
| 29 | 3.0 | 67.7 | 0.0084 | 152 | 106 | 30.2 | 0.570 | 72.9 |
| 30 | 3.45 | 68 | 0.0097 | 174 | 84 | 51.7 | 0.661 | 63.3 |
| 56 | 2 55 | 44 | 0.0104 | 216 | 156 | 27.8 | 0.460 | 84.2 |
| Mean | | | | | | | 0 474 | 75.9 |
| Standard error | | | | | | | ±0.031 | ±1.7 |

In the experiment presented in figure 1 the first ectopic beats were observed after the infusion of 78.6% of the fatal dose, the normal sinus rhythm was abolished, ventricular tachycardia being responsible for the steep rise of the recorded heart frequency.

The dose of digitoxin injected until the occurrence of the first ectopic beat—"Cardiac Irregularity Dose,"[2] expressed in per cent of the fatal dose—bears a remarkably constant relation to the fatal dose. Under sodium pentobarbital anesthesia (table 1) the cardiac irregularity dose is 74.5% with a standard error of only ±1.7 (11 experiments), whereas the standard error of the fatal dose

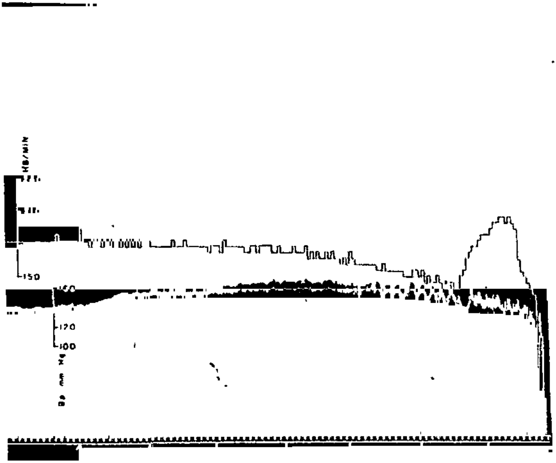

FIG 1 INFUSION OF DIGITOXIN

Cat, 2.45 k : Heart rate, beats per minute. intervals 30 seconds. Beginning of arrow Fatal dose 0.478 mgm. pc the fatal dose

(0.507 mgm. per kgm.) is relatively much larger (±0.042). In the series of experiments carried out in urethane-chloralose anesthesia, the same relation between the occurrence of irregularities and the fatal dose was found (table 2), the "cardiac irregularity dose" representing 75.9 (±1.7)% of the fatal dose of 0.474 mgm. per kgm. (±0.031).

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[2] This term, defining the minimum dose causing cardiac irregularities, has been used by Moe and Visscher (5) in a similar sense.

The transition from the sinus rate to an irregular rhythm was in most experiments sudden and abrupt. Made distinctly audible by the heart beat counter and visible on the fluorescent screen of the electrocardiograph, the beginning of the irregularity could be precisely timed. In no case was a return to a regular sinus rhythm observed after the occurrence of the first heterotopic ventricular beats. No detailed analysis was made of the character of the irregular heart rate. Ventricular tachycardia with frequencies from 250 to 300 was most frequently observed, in other experiments the frequency fell below the slowest sinus rhythm. The sudden cessation of the heart beat recorded by the heart beat counter afforded a sharp end point for the titration of the fatal dose. On inspection, the heart was always found in systolic contraction.

TABLE 3

*Infusion of ouabain in cats anesthetized with sodium pentobarbital*

| NO. | WEIGHT OF ANIMAL | DURATION OF INFUSION | RATE OF INFUSION | SLOWEST SINUS RATE | | DECREASE IN SINUS RATE | FATAL DOSE | CARDIAC IRREGULARITY DOSE |
|---|---|---|---|---|---|---|---|---|
| | | | | *Before Infusion* | *During infusion* | | | |
| | *kgm.* | *min.* | *mgm./min./kgm.* | | | *per cent* | *mgm./kgm.* | *Per cent of fatal dose* |
| 31 | 3.10 | 75.5 | 0.0021 | 220 | 120 | 45.4 | 0.157 | 51 |
| 33 | 3.35 | 52 | 0.0022 | 222 | 194 | 12.5 | 0.113 | 46.2 |
| 34 | 2.45 | 47 | 0.0026 | 165 | 165 | 0 | 0.129 | 63 |
| 35 | 2.45 | 70.3 | 0.0021 | 180 | 141 | 21.7 | 0.149 | 59.7 |
| 36 | 2.95 | 48.4 | 0.0023 | 218 | 180 | 17.4 | 0.112 | 61.1 |
| 37 | 3.35 | 53 | 0.0023 | 200 | 140 | 30.0 | 0.122 | 68.2 |
| 38 | 2.40 | 47.4 | 0.0026 | 204 | 116 | 43.1 | 0.121 | 58.2 |
| 39 | 2.45 | 47.2 | 0.0025 | 232 | 190 | 18.1 | 0.129 | 64.7 |
| 55 | 2.55 | 25.2 | 0.0022 | 264 | 220 | 16.7 | 0.101 | 65.5 |
| 57 | 2.05 | 43.7 | 0.0026 | 198 | 174 | 12.0 | 0.113 | 63.2 |
| 59 | 3.54 | 45 | 0.0023 | 210 | 188 | 10.5 | 0.102 | 68.8 |
| 60 | 3.20 | 36.8 | 0.0025 | 192 | 150 | 21.9 | 0.092 | 52.2 |
| Mean | | | | | | | 0.119 | 60.4 |
| Standard error | | | | | | | ±0.005 | ±2.07 |

*Infusion of ouabain.* Infusion of ouabain was carried out in two series of experiments under conditions comparable with those of the digitoxin experiments. The data from 12 experiments in sodium pentobarbital anesthesia and from 10 experiments in urethane-chloralose anesthesia are presented in tables 3 and 4. Infusion of ouabain produced a decrease in the heart rate which as a rule began earlier than that observed with digitoxin. In the experiment shown in figure 2, a gradual decrease was already noticed after the infusion of 15% of the fatal dose. However, except for the smaller amount of ouabain causing onset of the bradycardia, no difference was found between ouabain and digitoxin in their effect in slowing the sinus rate. A comparison of the data presented in tables 1 to 4 shows that wide variations in the degree of lowering of the heart rate were found with ouabain and digitoxin alike, irrespective of the type of

## TABLE 4

*Infusion of ouabain in cats anesthetized with urethane-chloralose*

| NO. | WEIGHT OF ANIMAL | DURATION OF INFUSION | RATE OF INFUSION | SLOWEST SINUS RATE | | DECREASE IN SINUS RATE | FATAL DOSE | CARDIAC IRREGULARITY DOSE |
|---|---|---|---|---|---|---|---|---|
| | | | | Before Infusion | During Infusion | | | |
| | *kgm.* | *min* | *mgm./min./kgm.* | | | *per cent* | *mgm./kgm.* | *per cent of fatal dose* |
| 3 | 3.00 | 43.8 | 0.0022 | 280 | 248 | 11.4 | 0.097 | 58.1 |
| 40 | 2.55 | 36.4 | 0.0025 | 206 | 171 | 16.0 | 0.091 | 59.1 |
| 41 | 2.55 | 40.5 | 0.0025 | 206 | 200 | 2.8 | 0.102 | 64.2 |
| 43 | 2.20 | 40.4 | 0.0024 | 231 | 166 | 28.1 | 0.098 | 51.1 |
| 44 | 2.65 | 38.7 | 0.0023 | 250 | 188 | 24.8 | 0.087 | 56.9 |
| 45 | 3.00 | 51.2 | 0.0020 | 220 | 192 | 12.7 | 0.102 | 66.0 |
| 50 | 3.00 | 48.8 | 0.0021 | 180 | 150 | 16.6 | 0.104 | 51.2 |
| 51 | 3.00 | 50 | 0.0021 | 180 | 158 | 12.2 | 0.105 | 55.0 |
| 53 | 2.70 | 46.8 | 0.0022 | 188 | 108 | 42.5 | 0.101 | 55.9 |
| 54 | 2.50 | 52 | 0.0020 | 182 | 136 | 25.2 | 0.106 | 66.2 |
| Mean | | | | | | | 0.099 | 58.4 |
| Standard error | | | | | | | ±0.002 | ±1.8 |

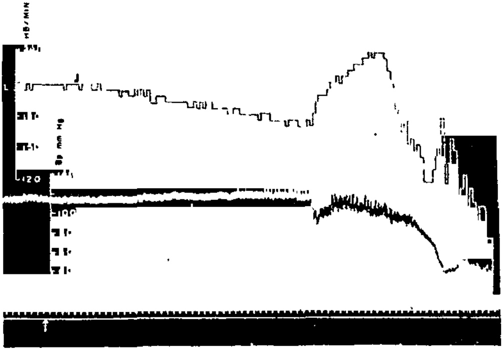

FIG. 2. INFUSION OF OUABAIN

Cat 2.55 k Heart rate, beats per minute. intervals 30 seconds Beginning of arrow Fatal dose. 0.091 mgm. pe the fatal dose anesthesia. An arithmetical average of the decrease in heart rate does not reveal a significant difference between the action of the two glucosides.

As with digitoxin, the slowest sinus rate was recorded in the majority of the experiments at the moment when the first ectopic beats were observed, and abrupt and complete transition from the sinus rhythm to heterotopic ventricular arrhythmia was the rule. In the experiment presented in figure 2 the arrhythmia began with a marked ventricular tachycardia comparable with that observed with digitoxin in figure 1. However, irregularity of the heart beat, coinciding with a transient fall in blood pressure in figure 2, occurred at 59.1% of the fatal dose. The "cardiac irregularity dose" of ouabain in the 22 experiments listed in tables 3 and 4, ranged from 46.2% to 68.8% of the fatal dose and was strikingly lower than the irregularity dose found in the 21 experiments with digitoxin (tables 1 and 2). The mean cardiac irregularity dose of ouabain was 60.4% (±2.07) of the fatal dose under sodium pentobarbital anesthesia and 58.4% (±1.8) under urethane-chloralose anesthesia. The consistency of these findings, indicated by comparatively small deviation of the mean, was comparable to the findings with digitoxin. The significance of the differences found in the four series of experiments is shown in table 5. A significance ratio[3] of 0.73 and 0.58 respectively between the two series with either glucoside bears evidence that the occurrence of irregularities is not significantly influenced

TABLE 5

*Difference between the cardiac irregularity doses of ouabain and digitoxin*

$$\text{Significance ratio} = \frac{\text{Difference}}{\text{Error of difference}}$$

| | | |
|---|---|---|
| OUABAIN<br>Urethane-chloralose<br>58.4% ±1.8 | Significance ratio<br>⟵⟶<br>0.73 | OUABAIN<br>Sodium pentobarbital<br>60.4% ±2.07 |
| ↑<br>Significance ratio<br>7.07<br>↓ | | ↑<br>Significance ratio<br>5.27<br>↓ |
| 75.9% ±1.7<br>DIGITOXIN<br>Urethane-chloralose | Significance ratio<br>⟵⟶<br>0.58 | 74.5% ±1.7<br>DIGITOXIN<br>Sodium pentobarbital |

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[3] The significance ratio is a measure of the probability that the observed difference is not due to chance. The odds against the occurrence of a given difference being due to chance in random sampling for a given significance ratio are tabulated. The values for the ratios calculated are:

| *Significance ratio* | *Odds against chance occurrence* |
|---|---|
| 0.58 | less than 1 to 1 |
| 0 73 | about 1 to 1 |
| 5 27 | over 1 774 000 to 1 |
| 7 07 | over 400 000 000 000 to 1 |

by the type of anesthesia employed. On the other hand, a significance ration of 7.07 and 5 27 respectively between the cardiac irregularity doses of ouabain and of digitoxin with different anesthetic agents establishes the reality of this difference.

*Influence of atropine upon the infusion of digitoxin and of ouabain.* In an attempt to analyze the difference in the cardiac irregularity dose between digitoxin and ouabain, atropine (2 mgm. per cat) was administered intravenously to the animals prior to the infusion of the glucoside. Tables 6 and 7 present the data obtained in a series of 10 and 12 experiments respectively carried out with digitoxin and ouabain in sodium pentobarbital anesthesia under conditions otherwise identical with those in the previous experiments.

TABLE 6

*Infusion of digitoxin following premedication with atropine (2 mgm ) in cats anesthetized with sodium pentobarbital*

| NO | WEIGHT OF ANIMAL | WEIGHT OF HEART | DURATION OF INFUSION | RATE OF INFUSION | SLOWEST SINUS RATE | | DECREASE IN SINUS RATE | FATAL DOSE | CARDIAC IRREGULARITY DOSE |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Before infusion | During infusion | | | |
| | *kgm* | *grams* | *min.* | *mgm /min /kgm* | | | *per cent* | *mgm /kgm* | *per cent of fatal dose* |
| A-14 | 3.16 | 14.23 | 46 | 0.0112 | 180 | 144 | 20 0 | 0.514 | 84 0 |
| A-15 | 2 65 | 9.90 | 63 | 0 0116 | 182 | 150 | 17 5 | 0 732 | 81.8 |
| A-16 | 2 35 | 9.75 | 49.5 | 0.0126 | 170 | 146 | 14 0 | 0.622 | 76.8 |
| A-17 | 2.93 | 15 40 | 34 | 0.0121 | 216 | 230 | 6 5 | 0 410 | 76 5 |
| A-18 | 2.70 | 11 40 | 51 5 | 0 0115 | 192 | 146 | 23.9 | 0 591 | 71.8 |
| A-21 | 2 93 | 13 30 | 66.4 | 0 0121 | 152 | 136 | 10 5 | 0.805 | 76.8 |
| A-23 | 3 49 | 14.30 | 46.5 | 0 0116 | 190 | 156 | 17.8 | 0.540 | 76 3 |
| A-24 | 2 60 | 11 40 | 40.2 | 0 0135 | 170 | 148 | 12 0 | 0 542 | 75.9 |
| A-25 | 2 90 | 11.70 | 39 | 0 0137 | 154 | 125 | 18 8 | 0.533 | 78.2 |
| A-26 | 3 17 | 14 60 | 41 | 0 0125 | 152 | 142 | 6 5 | 0.514 | 72 0 |
| Mean . . | | | | | | | . | 0.580 | 77.0 |
| Standard error | | | | . | | | | ±0 036 | ±1.2 |

The intravenous injection of 2 mgm. of atropine produced a moderate increase in the heart rate, as recorded in figure 3, and practically no effect upon the blood pressure when given slowly. The decrease in the heart rate following digitoxin was not significantly influenced by atropine, although the variations in the degree of the bradycardia (table 6) were somewhat smaller than in the experiments without atropine. Likewise, the decrease in heart rate following ouabain was practically unchanged by the injection of atropine. However, its early onset shown in figure 2 appeared to be delayed by atropine (fig. 3) and the progress of the slowing of the sinus rhythm in these experiments became comparable to that observed with digitoxin (fig. 1).

The "cardiac irregularity dose" of digitoxin in the atropinized cat (77.0% ± 1.2, table 6) was not significantly different from that found without atropine

(74.5% ± 1.7, table 1). On the other hand, premedication with 2 mgm. of atropine increased the "cardiac irregularity dose" of ouabain markedly from 60.4% ± 2.07 (table 3) to 75.8% ± 1.4 (table 7), almost exactly the figure found with digitoxin. Thus atropine, by preventing the early occurrence of irregularities found characteristic of ouabain in our experiments, abolished the differences in the cardiac action between the two glucosides. This indicates a difference in the vagal effect between ouabain and digitoxin.

Discussion. The determination of the lethal dose by the Hatcher method followed in general the modifications of de Lind van Wijngaarden (8), except that infusion from a burette was replaced by continuous injection from a pump and that sodium pentobarbital or a combination of urethane and chloralose was used instead of ether. The mean lethal doses of 0.474 and 0.507 mgm. per kgm. obtained with digitoxin in the two series of experiments are significantly higher than those reported by Haag and Woodley (9) and Weese (10) (0.318 and 0.33 mgm. per kgm. respectively), but in better agreement with the values found by Fromherz and Welsch (11), Rothlin (12) and Walther (6) (0.41, 0.42 and 0.47 mgm. per kgm. respectively)[4]. The lethal doses of 0.099 and 0.119 mgm. per kgm. found for ouabain agree closely with the values determined by Edmunds and coworkers (13), Haag and Woodley (9), Lieb and Mulinos (14), Fromherz

TABLE 7

*Infusion of ouabain following premedication with atropine (2 mgm.) in cats anesthetized with sodium pentobarbital*

| NO. | WEIGHT OF ANIMAL | WEIGHT OF HEART | DURATION of INFUSION | RATE OF INFUSION | SLOWEST SINUS RATE |  | DECREASE IN SINUS RATE | FATAL DOSE | CARDIAC IRREGULARITY DOSE |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | Before Infusion | During Infusion |  |  |  |
|  | *kgm.* | *grams* | *min.* | *mgm./min./kgm.* |  |  | *per cent* | *mgm./kgm.* | *per cent of fatal dose* |
| A-1 | 2.80 | 11.71 | 48.2 | 0.0019 | 182 | 182 | 0 | 0.091 | 86.1 |
| A-2 | 2.45 | 10.45 | 44 | 0.0022 | 228 | 194 | 14.9 | 0.098 | 75.0 |
| A-3 | 2.29 | 10.22 | 59.8 | 0.0022 | 160 | 152 | 5.0 | 0.130 | 73.4 |
| A-4 | 2.18 | 10.25 | 52 | 0.0023 | 198 | 156 | 21.2 | 0.119 | 78.0 |
| A-5 | 3.00 | 14.00 | 44.3 | 0.0021 | 216 | 164 | 24.0 | 0.092 | 74.6 |
| A-8 | 3.50 | 17.30 | 61.5 | 0.0019 | 156 | 138 | 11.5 | 0.117 | 73.2 |
| A-9 | 3.16 | 15.45 | 57.8 | 0.0023 | 160 | 114 | 28.7 | 0.133 | 71.5 |
| A-10 | 2.71 | 10.45 | 45.3 | 0.0023 | 170 | 152 | 10.5 | 0.103 | 73.5 |
| A-12 | 2.96 | 12.96 | 49.8 | 0.0023 | 170 | 144 | 15.3 | 0.113 | 72.3 |
| A-13 | 3.50 | 15.65 | 52.8 | 0.0020 | 186 | 140 | 24.6 | 0.107 | 81.3 |
| A-27 | 2.72 | 10.80 | 52.8 | 0.0023 | 160 | 128 | 20.0 | 0.119 | 73.5 |
| A-28 | 3.10 | 15.80 | 39.8 | 0.0024 | 258 | 234 | 9.3 | 0.096 | 74.5 |
| Mean |  |  |  |  |  |  |  | 0.110 | 75.8 |
| Standard error |  |  |  |  |  |  |  | ±0.004 | ±1.4 |

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[4] The variations in the lethal doses reported by the various investigators may have been influenced by the use of digitoxin from different sources.

and Welsch (11), Lendle (15), Rothlin (12) and Weese (10), ranging from 0.098 to 0.118 mgm. per kgm. and obtained in many instances under somewhat different conditions.

In our experiments the standard error of the fatal dose of digitoxin (8.3%, 6.5%) and particularly of ouabain (2%, 4.2%), is considerably smaller than that reported by or calculated from the data of most other investigators. It would seem that the use of a pump to secure an uninterrupted and constant rate of injection contributes towards a low standard error. Another possible factor in reduction of the standard error is the anesthetic agent. Ether, probably still the most widely used anesthetic in digitalis assays on cats, was used in the majority of investigations cited above. An excellent critique of its use in digitalis assays has been offered by Burn (16). It has been demonstrated repeatedly that the variation of the lethal dose in cats is distinctly smaller when a non-volatile anesthetic such as chloralose (17, 18), dial (17), paraldehyde (18, 19), chloretone (18), or urethane (18) is used instead of ether; spinal cats were found to yield even more satisfactory results (20, 21, 27). Our experience with sodium pentobarbital and particularly with urethane-chloralose tend to support the evidence

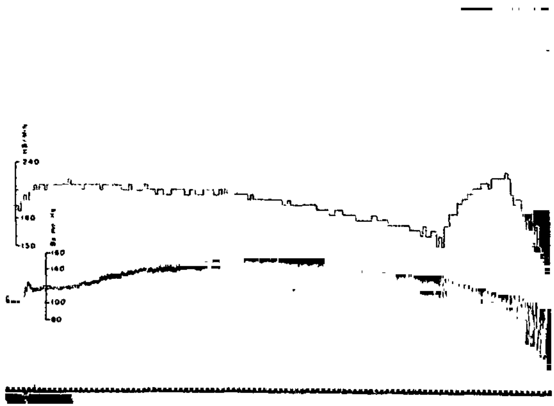

FIG. 3. INFLUENCE OF ATROPINE UPON THE INFUSION OF OUABAIN

Cat 3 kgm. Sodium pentobarbital anesthesia Upper tracing Heart rate, beats per minute. Lower tracing Carotid blood pressure, mm Hg. Time intervals 30 seconds. Injection of 2 mgm of atropine sulfate at 1. Beginning of infusion of 0.0021 mgm. per kgm per min. indicated by arrow Fatal dose 0.092 mgm per kgm. (44.3 min.). Cardiac irregularity dose 74.6% of the fatal dose.

brought forward by Macdonald (21) and others in favor of a non-volatile anesthetic agent over ether.

The decrease in the sinus rate following the injection of either digitoxin or ouabain varied considerably. An average decrease of about 20% in the frequency was recorded in the majority of the experiments with digitoxin as well as with ouabain. The concomitant changes in the blood pressure were slight and variable; no correlation between the decrease in heart rate and the changes in blood pressure could be established.

Differences between digitoxin and ouabain were found in the onset of the decrease in the sinus rhythm. In the experiments with digitoxin slowing of the heart rate became noticeable after the infusion of about 30% of the fatal dose. This finding agrees well with the observations of Bauer and Reindell (22) and Walther (6); Robinson and Wilson (23), using tincture of digitalis, likewise observed the beginning of bradycardia after 30% of the lethal dose. In our experiments with ouabain, on the other hand, slowing of the heart rate began in the majority of the experiments after only about 15% of the fatal dose had been given.

Irregularity of the heart beat was produced with 60% of the fatal dose of ouabain, whereas with digitoxin cardiac irregularities were not observed until 75% of the fatal dose was administered. The data presented in this paper demonstrate a consistent relationship between the occurrence of ectopic beats and the fatal dose, and a significant difference in this relationship between digitoxin and ouabain. Following the infusion of digitoxin under ether anesthesia, beginning of ventricular rhythm has been found by Planelles and Werner (23) after 70%, by Bauer and Reindell (22) after 68%, and by Walker (6) after 61% of the fatal dose. These authors observed the early occurrence of occasional extrasystoles and a gradual change to a ventricular rhythm, whereas in our experiments the transition from sinus rhythm to heterotopic ventricular rhythm was sharply defined. Gold and coworkers (3, 4) in experiments conducted with local rather than general anesthesia failed to obtain evidence for a significant difference in the occurrence of ectopic beats between digitoxin and ouabain. They state that the early appearance of ectopic beats was often due to struggling. In view of the wide variations of their results, probably due to the technique their evidence against a possible difference in the cardiac action between digitoxin and ouabain appears hardly conclusive.

Atropinization of the animal did not cause a systematic change in the fatal doses of digitoxin and ouabain for the value for digitoxin increased, whereas that for ouabain was slightly decreased. Lendle (25) reported a slight decrease in toxicity both of digitoxin and ouabain in atropinized cats anesthetized with tribromethanol. In our experiments with atropine, both digitoxin and ouabain produced slowing of the heart rate to a degree comparable to that observed without atropine. However, the early onset of the bradycardia observed with ouabain was delayed when atropine had been given.

Cardiac irregularities following digitoxin occurred at practically the same fraction of the lethal dose in both series of experiments, with an without atropine.

On the other hand, the "cardiac irregularity dose" of ouabain was regularly and significantly increased in animals which received atropine. The onset of cardiac irregularities after the infusion of 75.8% of the fatal dose of ouabain in the atropinized cat corresponds well with the findings of Hoekstra and Schleusing (26) on vagotomized cats. These authors reported that sudden and sharply defined change from sinus rhythm to a heterotopic ventricular rhythm occurred after the infusion of 75% of the fatal dose of ouabain in 5 cats anesthetized with tribromethanol.

In the cats premedicated with 2 mgm. of atropine, cardiac irregularities due to the infusion of ouabain were found at the same percentage of the lethal dose as with digitoxin, and no striking difference in the character of these irregularities (heterotopic ventricular beats) was evidenced by the electrocardiogram. These findings permit the conclusion that the difference in the onset of cardiac irregularities between the two glucosides is due to a particular effect of ouabain upon the vagal nerve, which can be suppressed by atropine.

*Acknowledgment.* Appreciation is expressed to Dr. S. Feitelberg for the statistical interpretation of the results and to Mr. J. G. Greslin for valuable technical assistance.

### SUMMARY

The toxic effects of digitoxin and ouabain were studied in 65 cats anesthetized with sodium pentobarbital or with urethane-chloralose.

1. A consistent relationship was found between the appearance of cardiac irregularities and the fatal dose of each glucoside. Cardiac irregularities occurred after the infusion of about 75% of the fatal dose of digitoxin; with ouabain, they appeared after the injection of about 60% of the fatal dose.

2. This difference in the cardiac action between digitoxin and ouabain was found to be the same regardless of the type of anesthesia employed.

3. Atropine delayed the occurrence of irregularities following ouabain until about 75% of the fatal dose had been injected; it did not influence significantly the occurrence of irregularities produced by digitoxin.

4. The results are interpreted as evidence for a difference in the cardiac action between digitoxin and ouabain in cats. Ouabain exerts an effect upon the vagus which can be suppressed by atropine; digitoxin does not have this effect.

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# FEDERATION PROCEEDINGS

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# THE LOCAL ANESTHETIC PROPERTIES OF CINNAMYLEPHEDRINE[1]

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In a previous paper (1) the action of cinnamylephedrine on smooth muscle has been described. Although Ehrhart (2) reported that cinnamylephedrine possessed local anesthetic properties, he published no quantitative data. It seemed desirable therefore to make a quantitative study comparing the *d*, *l*, and racemic forms of cinnamylephedrine with well established local anesthetics such as procaine and cocaine.

The usual laboratory procedures were used in evaluating the cinnamylephedrines as local anesthetics.

I. RABBIT'S CORNEA. Surface anesthetic potency was determined by the rabbit's cornea method as employed by Sollmann (3). The rabbit was placed in a wooden box with its head protruding at one end. The eyelashes were clipped A solution of the drug to be examined was placed, by means of an eye dropper, into a pocket formed by pinching the lower eyelid. The pocket thus formed was flooded with the solution for one minute. The animal was then released. The wink reflex was determined by bringing the dull point of a pencil along the side of the head and touching the cornea in several places. Absence of the wink reflex was taken as evidence of anesthesia.

If the first application did not cause complete anesthesia, the conjunctival sac of the animal was filled again, but this time the animal was released immediately and not held for one minute. The duration of anesthesia was considered as the time from the last application until the wink reflex completely returned to normal. The compounds, in the corneal tests, were compared with cocaine. All substances were in the form of the hydrochlorides and were dissolved in 0.73% NaCl.

The results are shown in table 1. The term "incomplete" signifies that anesthesia was present but submaximal. The minimal effective concentration of cocaine was found to be 0.5%, while the smallest concentration of the cinnamylephedrines found to produce surface anesthesia was 0.05%. All of the isomeric forms of cinnamylephedrine possessed the same minimal effective concentration, but the duration of action of the *d* form seemed to be somewhat longer than that of the *l* isomer. These data appear to show that the cinnamylephedrines are about ten times as potent as cocaine as surface anesthetics.

The cinnamylephredrine compounds caused considerable capillary engorgement of the palpebral conjunctiva and pitting of the cornea when used in concentrations of 1 and 2%, but concentrations smaller than 1% caused no detectable irritation. Ten per cent *l*-ephedrine possessed no surface anesthetic potency.

In the above experiments, although the usual mydriasis followed cocaine, no corresponding effect was observed after cinnamylephedrine.

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[1] This work was supported by the generous aid of the W. E. Weiss Fellowship Fund.

*II.* The human intradermal wheal. The procedure used was similar to that of Sollmann (4). The flexor surface of the forearm was washed with 70% ethyl alcohol and 0.4 cc. of a sterilized solution of the drug was injected intracutaneously. The anesthesia was tested by noting the loss of sensation to pin pricks as compared with surrounding area of the skin. The subject's head was always turned so that he could not see the area touched and he reported his sensations to the operator. The drugs were used in the form of the hydrochlorides and were dissolved in 0.73% NaCl. Procaine was used as the standard for comparison in the wheal tests.

TABLE 1

*Anesthesia of the rabbit's cornea*

| % concentration | cocaine HCl | | d-cinnamylephedrine HCl | | l-cinnamylephedrine HCl | | dl-cinnamylephedrine HCl | |
|---|---|---|---|---|---|---|---|---|
| | No. exper. | Aver. duration | No. exper. | Aver. duration | No. exper. | Aver. duration | No. exper. | Aver. duration |
| | | *minutes* | | *minutes* | | *minutes* | | *minutes* |
| 2 | 3 | 47 | 3 | 183 | 3 | 147 | | |
| 1 | 3 | 23 | 3 | 123 | 3 | 94 | | |
| 0.5 | 3 | 20 | 3 | 88 | 3 | 67 | | |
| 0.4 | 3 | incomplete | | | | | | |
| 0.25 | 3 | incomplete | 4 | 63 | 4 | 62 | | |
| 0.1 | 3 | none | 3 | 57 | 5 | 29 | | |
| 0.05 | | | 3 | 42 | 3 | 23 | 3 | 32 |
| 0.04 | | | 1<br>3 | 30<br>incomplete | 3 | none | 3 | incomplete |
| 0.025 | | | 3 | incomplete | 3 | none | 1<br>2 | incomplete<br>none |
| 0.01 | | | 3 | none | 3 | none | | |

The results are shown in table 2. In two human subjects, the minimal effective concentration of procaine was shown to be 0.05%. Under the same experimental conditions, the *l*-cinnamylephedrine and the *dl*-cinnamylephedrine produced local anesthesia in both subjects when concentrations as small as 0.005% were used, and in one subject when 0.0025% was injected. *d*-Cinnamylephedrine produced local anesthesia in both subjects when given in a concentration of 0.0025%. When 1:50,000 epinephrine HCl was added to either procaine or *d*-cinnamylephedrine, the duration of action was increased greatly, but the minimal effective concentrations were not altered.

One per cent solutions of the cinnamylephedrine compounds produced necrosis in the injected area, while the more dilute solutions produced varying degrees of erythema which persisted from twelve to twenty-four hours. No significant differences in the results were observed if the cinnamylephedrine HCl solutions were regulated to pH 7.4.

*l*-Ephedrine caused considerable irritation when it was injected, but nevertheless produced anesthesia in the higher concentrations, i.e. 0.1% or more. If *l*-ephedrine HCl solutions were adjusted to pH 7.4 the irritative effect was diminished, but duration and minimal effective anesthetic concentration were unchanged. The *l*-ephedrine solutions were tested for their purity polariscopically, and preparations from two different commercial houses were examined for their local anesthetic effects.

III. Anesthesia of the motor nerve of the frog. The excised sciatic nerve and gastrocnemius muscle were placed in troughs which were cut in a paraffin block. Ringer's solution bathed the preparation with the exception of a short length of the nerve trunk which was immersed in a solution of the drug to be tested. The excitability of the preparation was tested by applying to the distal end of the nerve, a simple break shock from the secondary coil of a Harvard Inductorium which was set at 12 cm. When the preparation failed to respond to the above stimulus, the time was recorded and the block was considered to be complete. The anesthesia of the motor nerve was found to be reversible. All compounds were compared with cocaine by using paired sciatic-gastrocnemius preparations from the same frog. The hydrochlorides of the compounds studied were dissolved in 0.7% NaCl.

TABLE 2

*Anesthesia from human intradermal wheal*

| % CONC. | WITHOUT EPINEPHRINE HCl | | | | | | WITH 1:50,000 EPINEPHRINE HCl | |
|---|---|---|---|---|---|---|---|---|
| | Duration procaine HCl | Duration CED HCl* | Duration CEL HCl† | Duration CER HCl‡ | Duration ephedrine HCl | | Duration procaine HCl | Duration CED HCl* |
| | | | | | Sample I | Sample II | | |
| | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* |
| 1.0 | 18<br>22 (20) | >200<br>>200 | | | | | | |
| 0.5 | 17<br>18 (17½) | >200<br>180 | | | 18<br>17 (17½) | 22<br>19 (20½) | >200<br>>200 | >200<br>>200 |
| 0.1 | 7<br>10 (8½) | 37<br>29 (33) | | | 2<br>8 (5) | 2<br>9 (5½) | 63<br>116 (94½) | 90<br>139 (114½) |
| 0.05 | 7<br>8 (7½) | 16<br>31 (23½) | | | 0<br>0 | 0<br>0 | 20<br>27 (23½) | 135<br>140 (137½) |
| 0 025 | 0<br>0 (0) | 18<br>25 (22½) | 13<br>20 (16½) | 25<br>21 (23) | | | 0<br>0 (0) | 57<br>110 (83½) |
| 0.01 | 0<br>0 | 12<br>21 (16½) | 7<br>15 (11) | 12<br>13 (12½) | | | | 60<br>82 (71) |
| 0.005 | | 7<br>10 (8½) | 6<br>6 (6) | 8<br>10 (9) | | | | 13<br>19 (16) |
| 0.0025 | | 2<br>4 (3) | 0<br>3 (1½) | 0<br>4 (2) | | | | |
| 0.001 | | 0<br>0 | 0<br>0 | 0<br>0 | | | | 0<br>0 |

* Cinnamylephedrine dextro.

† Cinnamylephedrine levo.

‡ Cinnamylephedrine racemic.

The results are shown in table 3. One per cent solutions of cocaine, *d*, *l*, and *dl*-cinnamylephedrine, and 5% *l*-ephedrine effectively blocked the sciatic nerve of the frog. The onset of anesthesia varied considerably in the various preparations and conclusions of a quantitative nature cannot be drawn from these data.

IV. Spinal anesthesia in frogs. The technique of Bieter, Harvey, and Burgess (5) for intraspinal injections in frogs was used in this study.

The hydrochlorides of the compounds were dissolved in 0.74% NaCl. The concentrations of the solutions injected were arranged so that volumes injected were no less than 0.03 cc. or no greater than 0.15 cc. Injections were made with a tuberculin syringe fitted with a twenty-seven gauge needle. Each frog received no more than one injection. The failure of the frog to withdraw its feet from 0.5 per cent hydrochloric acid within thirty seconds was considered indicative of spinal anesthesia.

Male winter frogs (Rana pipiens) kept in a tank at about 10°C, were used in this work. After injection the frogs were placed in a quarter of an inch of running water at about 15°C. No attempt was made to control posture.

TABLE 3

*Onset of anesthesia in frog's motor nerve*

| 1% CEL* | 1% COCAINE | 1% CED† | 1% COCAINE | 1% CER‡ | 1% COCAINE | 5% EPHEDRINE | 1% COCAINE |
|---|---|---|---|---|---|---|---|
| *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* |
| 32 | 23 | 24 | 39 | 28 | 31 | 37 | 48 |
| 37 | 49 | 34 | 43 | 43 | 43 | 37 | 28 |
| 5 | 13 | 37 | 37 | 42 | 66 | 36 | 14 |
| 14 | 22 | 30 | 28 | 45 | 35 | 57 | 50 |
| 23 | 37 | 30 | 42 | 32 | 45 | 26 | 52 |
| Av. . 22 | 29 | 31 | 38 | 38 | 44 | 48 | 48 |

* Cinnamylephedrine *Levo*.
† Cinnamylephedrine *Dextro*.
‡ Cinnamylephedrine Racemic.

The results are shown in table 4. The minimal anesthetic dose, or the dose which anesthetized 50% of the animals, was about 0.015 mgm. per gram for procaine, and about 0.01 mgm. per gram for *d*-cinnamylephedrine and *l*-cinnamylephedrine. There were no striking differences in the onset and duration of the anesthesia produced by the above compounds.

It has been shown elsewhere *l*-ephedrine also effects nerve conduction when injected intraspinally (6).

All animals recovered from the injections. When the higher doses were injected it was not possible to determine the exact recovery time, however, reflex tests made about twelve hours after the injections indicated that the animal had completely recovered.

V. Toxicity and symptoms of cinnamylephedrine in mice. Toxicity determinations were made on male and female gray mice of the Strong CHI strain. These animals, weighing between 16 and 25 grams, had been kept on a uniform diet preceding experimentation, and then were fasted 24 hours before the subcutaneous injections. Both *l*- and *d*- forms were investigated; the results are shown in table 5.

Evidently for mice the subcutaneous LD 50 of *l*-cinnamylephedrine is approximately 150 mgm./kg., while the *d*- form exhibits an LD 50 of about 75 mgm./kg. It is interesting to note that although the *l* isomer shows somewhat greater spasmolytic and depressor effects than the *d* isomer (1), the latter is twice as toxic.

TABLE 4

*The anesthetic effect of intraspinal cinnamylephedrine HCl and procaine HCl on the frog*

| DRUG | DOSE | NO OF ANIMALS | % ANESTHETIZED | AVER. ONSET ANESTHESIA | AVER. DURATION OF ANESTHESIA |
|---|---|---|---|---|---|
| | *mg /gm* | | | *minutes* | *minutes* |
| Procaine | 0.010 | 8 | 12.5 | 16 | 10 |
| | 0.015 | 9 | 44 | 6 | 13 |
| | 0.018 | 9 | 67 | 4 | 54 |
| | 0.020 | 9 | 78 | 9 | 35 |
| | 0.030 | 10 | 89 | 5 | 36 |
| | 0.045 | 7 | 100 | 3 | 45 |
| *l*-Cinnamylephedrine | .005 | 8 | 0 | | |
| | 008 | 8 | 25 | 4 | 74 |
| | .01 | 8 | 50 | 8 | 60 |
| | 02 | 8 | 89 | 5 | 98 |
| | 03 | 8 | 100 | 4 | 160 |
| *d*-Cinnamylephedrine | .005 | 8 | 12.5 | 5 | 13 |
| | .008 | 8 | 12 5 | 19 | 9 |
| | .01 | 8 | 62 5 | 15 | 50 |
| | 02 | 8 | 75 | 8 | 91 |
| | .03 | 8 | 100 | 3 | 200 |

TABLE 5

*Toxicity of l- and d-cinnamylephedrine for mice*

| DOSE | LEVO- FORM | | DEXTRO- FORM | |
|---|---|---|---|---|
| | No of mice | Fatality | No. of mice | Fatality |
| *mgm /kilo* | | % | | % |
| 50 | 5 | 0 | 14 | 0 |
| 60 | | | 20 | 20 |
| 75 | | | 33 | 48 |
| 100 | 19 | 11 | 30 | 70 |
| 135 | 30 | 23 | | |
| 150 | 30 | 53 | 12 | 75 |
| 180 | | | 7 | 100 |
| 250 | 6 | 83 | | |
| 300 | 6 | 83 | | |

Following the injections the animals showed general depression, unsteady gait, and later hyperexcitability and clonic convulsions after which they either recovered or showed signs of central nervous system depression and died. Convulsions usually preceded death. The *d*- form produced convulsions which were of a more rapid onset and a longer duration than the *l*- form. General depression, which usually preceded the convulsions, was more prominent in the animals injected with the levo- form. In many instances with both forms of the drug, the tails of the mice were erected, as after morphine (Straub effect).

DISCUSSION. The local anesthetic properties of ephedrine were demonstrated in an earlier paper (6).

Derivatives of cinnamic acid and cinnamic alcohol have been shown to possess local anesthetic properties (4, 7, 8, 9). Read (10) has shown that benzyl ephedrine is about twice as active as procaine when tested by the intradermal wheal method. Cinnamylephedrine resembles benzylephedrine in that it combines ephedrine with an aryl group which has been shown to possess local anesthetic properties. When the results shown here are compared with those of Read with benzylephedrine it appears that cinnamylephedrine is much more potent than benzylephedrine as a local anesthetic, since the data shown in table 3 indicate that cinnamylephedrine is about ten times as potent as procaine.

It is of considerable interest to note that the *d*- form of cinnamylephedrine produces corneal anesthesia of longer duration than the *l*- form, although the latter has a greater depressant effect on smooth muscle (1).

## SUMMARY

1. Cinnamylephedrine (*d*, *l*, and *dl*) is about ten times as potent a surface anesthetic as cocaine when tested by the rabbit's cornea method. Irritation occurs when concentrations of one per cent or greater are used.

2. When tested by the human intradermal wheal method, cinnamylephedrine (*d*, *l*, and *dl*) is about twenty times as potent as procaine. The compound is somewhat irritating and causes an erythema which persists from twelve to twenty-four hours.

3. One per cent cinnamylephedrine (*d*, *l*, and *dl*) is capable of blocking the motor nerve of the frog.

4. Cinnamylephedrine (*d* and *l*) produces spinal anesthesia in frogs, being slightly more active than procaine in this respect.

5. *l*-Cinnamylephedrine is about one-half as toxic for mice as the *d*- form.

The helpful advice of Dr. H. G. Barbour is gratefully acknowledged.

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# SOME NEW ASPECTS OF MORPHINE ACTION: EFFECT ON THE STOMACH[1, 2]

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The literature dealing with the effects of drugs on the stomach is rife with controversies. This work was undertaken in an attempt to explain some known effects of morphine and to present some new features of its action. It has been reported by Slaughter and Gross (1) that physostigmine potentiates the effect of morphine on the intestine of unanesthetized dogs, on blood pressure in cats and on toxicity in rats. Further, we (2) have shown that prostigmine methylsulfate potentiates the effectiveness of morphine in relieving pressure pain in cats. Winter (3) has reported effects of morphine on the bladder which correlate well with these results. Quite recently McCrea, *et al.* (4) pointed out that prostigmine methysulfate enhances morphine miosis. These findings indicate that morphine acts as a cholinergic drug and Wright (5) has shown that it depresses choline esterase *in vitro.* These experiments suggest that one might expect other actions of morphine to be potentiated by cholinergic drugs.

In this study we have shown that the effects of morphine on the stomach can also be potentiated by a cholinergic drug. Prostigmine methylsulfate was used as the potentiating drug because it probably has fewer untoward side reactions than does physostigmine. Healthy female dogs ranging from 9.5 to 13.2 kg. were operated upon and a Janeway gastrostomy was made using the upper portion of the stomach. These animals were used after complete recovery (usually two weeks) and at this time there was no leakage from the fistulae. The method of Plant and Miller (6) was adopted to record the stomach contractions of unanesthetized dogs. Each animal was used only once a week to preclude any possibility of morphine tolerance.

RESULTS. The results contained in this report are based on 77 observations on five dogs. The subcutaneous dose of prostigmine methylsulfate ranged from 0.025 to 0.06 mgm./kg., while intravenously 0.03 to 0.04 mgm./kg. was given. The subcutaneous dose of morphine sulfate employed was routinely 0.05 mgm./kg., except in a few instances where 0.1 mgm./kg. was used.

Before reporting the effects on the stomach, certain side reactions in these experiments following the administration of prostigmine methylsulfate are worth relating. With effective doses of prostigmine methylsulfate, skeletal muscle twitchings invariably occurred and usually accompanied the initial effects

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[1] This work was made possible by a grant to one of us (DS) from Hoffmann-La Roche, Inc , Nutley, New Jersey.

[2] A preliminary report was read before the Section of Gastro-Enterology and Proctology, American Medical Association Meeting, Cleveland, June 2–6, 1941.

on the stomach. When these twitchings were at their peak of activity the tonicity of the stomach was usually lowest. As has been reported by Necheles, *et al.* (7), increased gastric secretion from the fistulae occurred with subcutaneous doses of 0.06 and 0.05 mgm./kg. and intravenous doses of 0.03 mgm./kg. of prostigmine methylsulfate. Lacrimation also was often observed and a slight uterine discharge was some times seen. Defecation usually followed the larger subcutaneous dose as well as the intravenous dose and at times vomiting occurred. Also, following the administration of such doses the animals were quiet and appeared to be more drowsy than usual.

TABLE 1

*Analysis of results*

| NUMBER OF EXPERIMENTS | DRUG | DOSE | ROUTE OF ADMINISTRATION | "USUAL EFFECT" | PER CENT OF EXPERIMENTS CONFORMING TO "USUAL EFFECT" | LENGTH OF ACTION (AVERAGE) |
|---|---|---|---|---|---|---|
| | | *mgm./kg.* | | | | *minutes* |
| 12 | Prostigmine Methylsulfate | 0.05 | Subcutaneous | Decrease in amplitude and rate of contractions plus decrease in tonus | 91.7 | 90 |
| 9 | Prostigmine Methylsulfate | 0.03 | Intravenous | Rapid fall in tonus with complete cessation of contractions | 100 | 10–20 |
| 5 | Prostigmine Methylsulfate | 0.025 | Subcutaneous | No effect | 100 | |
| 5 | Morphine sulfate | 0.05 | Subcutaneous | No effect | 100 | |
| 14 | Prostigmine methylsulfate 0.025 mg./kg. followed in 10 minutes by morphine sulfate: | 0.05 | Subcutaneous | Marked fall in tonus plus decreased stomach motility followed by increased tone and amplitude of contractions | 92.8 | 35–45 |
| 8 | Prostigmine methylsulfate + morphine sulfate. Simultaneous administration | 0.025 + 0.05 | Subcutaneous | Fall in tonus plus a decrease in rate and amplitude of contraction | 87.5 | 35 |

In table 1 are recorded the results on the stomach of the "standard" doses employed. A typical tracing of these various effects is shown in figure 1. The effects of some of the miscellaneous dosages and dosage combinations are mentioned only in the text.

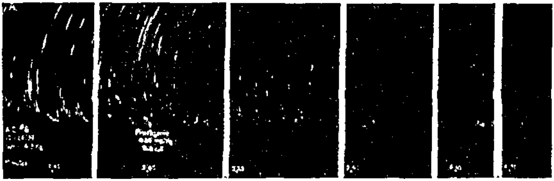


FIG. 1, A. A TYPICAL EFFECT OF SUBCUTANEOUS PROSTIGMINE METHYLSULFATE ON THE STOMACH

Note the reduction in number and amplitude of waves. Time trace, minutes

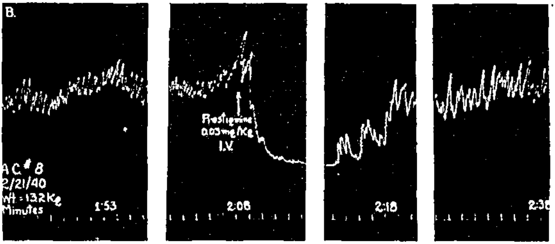


FIG. 1, B. A TYPICAL EFFECT OF INTRAVENOUS PROSTIGMINE METHYLSULFATE ON THE STOMACH

Note immediate, marked reduction in tonus with cessation of waves. Time trace, minutes

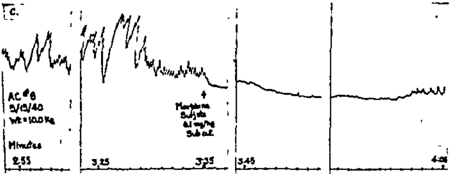
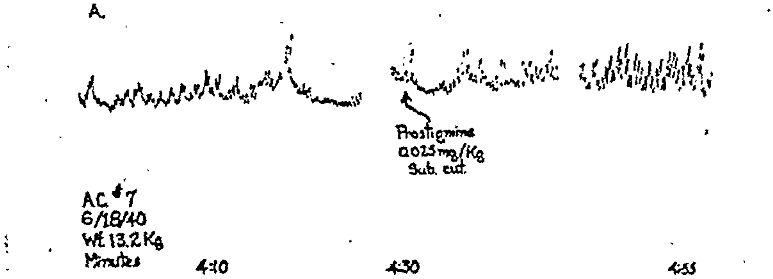


FIG 1, C. A TYPICAL EFFECT OF SUBCUTANEOUS MORPHINE ON THE STOMACH

Note similarity to tracing A Time trace, minutes

Trial experiments indicated that 0.06 mgm./kg. of prostigmine methylsulfate administered subcutaneously gave a consistent, marked decrease in tonus plus a decrease in the number of contractions. These effects lasted about one and one-half hours. However, unfavorable side reactions (lacrimation, intense fibrillary muscle twitching and defecation) led us to use a smaller dose (0.05 mgm./kg.) which gave a similar effect on the stomach, but did not produce as many untoward side effects. As a rule, this dose caused a decrease in the amplitude and rate of contractions plus some fall in tonus. Even if the tonus was not lowered appreciably, contractions were always decreased in amplitude and frequency. Figure 1, A shows a typical effect. The effect came on in seven minutes and lasted one and one-half hours. On occasion, following the period of decreased tonicity and amplitude of contractions, the return to normal was followed by an increase in amplitude of contractions. This was not a constant effect, however.



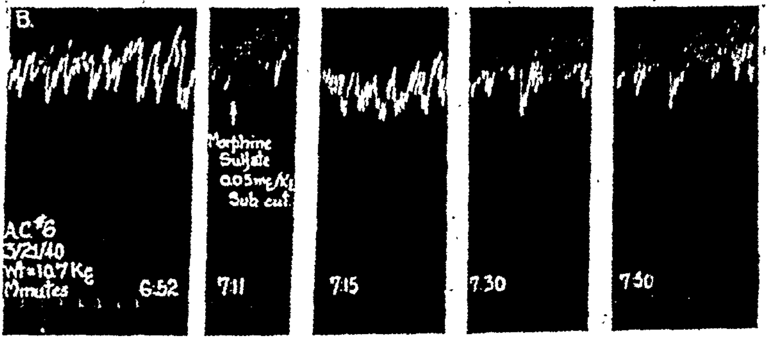


Fig. 2, A. Lack of Action on the Stomach when 0.025 mgm. kg. of Prostigmine Methylsulfate is Used Subcutaneously

*Time trace, minutes*

Fig. 2, B. Absence of Effect on the Stomach when a Subliminal Dose of Morphine Is Used

*Time trace, minutes*

The effects of intravenous administration of prostigmine methylsulfate were very interesting. In figure 1 B, 0.03 mgm./kg. produced a very rapid fall in tonus with complete cessation of contractions. These effects lasted about ten to twenty minutes with gradual return to normal. This result was extremely consistent and occurred whether the tonus of the stomach was high or low. This result is similar to that from an effective dose of morphine as regards depression of tonicity but the duration of this action is much less than that exhibited by morphine.

We felt, however, that a more valid basis for comparison with the effects of morphine might be afforded by the use of a larger *subcutaneous* dose of prostigmine methylsulfate (such as 0.05 mgm./kg.) since this dose produced a comparable depression of amplitude and of frequency over a much longer period of time.

The effects of morphine on the stomach have been well worked out by Plant and Miller (6). These authors state the minimal effective subcutaneous dose to be 0.1–0.25 mgm./kg. We found that 0.1 mgm./kg. gave consistent results and figure 1, C illustrates a typical response. As reported by Plant and Miller (6) this action consists of reduction in tonus and amplitude of contractions which lasts for several hours.

We were interested in obtaining information regarding subthreshold doses of prostigmine methylsulfate and morphine in order to test the potentiation aspect. Trial experiments showed 0.025 mgm./kg. of prostigmine methylsulfate and 0.05 mgm./kg. of morphine subcutaneously to be ineffective individually (see fig. 2, A and B). The ineffectiveness of this small dose of morphine corroborates the findings of Plant and Miller (6).

Potentiative effects of these drugs at the subliminal dose level are seen in figure 3, A and B. In A, the inactive subcutaneous dose of prostigmine methylsulfate was followed in ten minutes by the subliminal subcutaneous dose of morphine. Prostigmine methylsulfate alone caused no effect, but in about five minutes after the morphine was given, a typical morphine-like effect on the stomach occurred, i.e., marked fall in tonus and marked reduction of stomach motility. This lasted about thirty-five minutes. Following this effect, a marked increase in tone and amplitude of contractions is noted. This differs from the administration of simultaneously administered subliminal doses of prostigmine methylsulfate and morphine as seen in figure 3, B. Here the typical morphine-like action is noted, but no after increase in tone or amplitude is observed.

Veach, *et al.* (8), have reported that on the human stomach the inhibitory action of atropine is made motor by prostigmine methylsulfate. We therefore performed experiments to ascertain if such an effect occurs in the dog. We were unable to obtain results in our animals which indicated that atropine effects on the stomach were reversed by prostigmine methylsulfate.

DISCUSSION. The results clearly indicate that morphine acts on the stomach like a cholinergic drug and is potentiated by a known parasympathetic stimulant. That there is true potentiation is evidenced by the fact that small doses of prostigmine methylsulfate and morphine which have little or no effect when used singly, produce marked effects when given together. There are two differences in the action of small combined doses as compared with a larger (typical or active) dose of morphine alone. First, the period of hypotonicity and of obliterated contractions is much shorter. Secondly, the increased tonus following the morphine-like tonus reduction noted when a subliminal dose of prostigmine methylsulfate is followed by an inactive dose of morphine, is never seen with

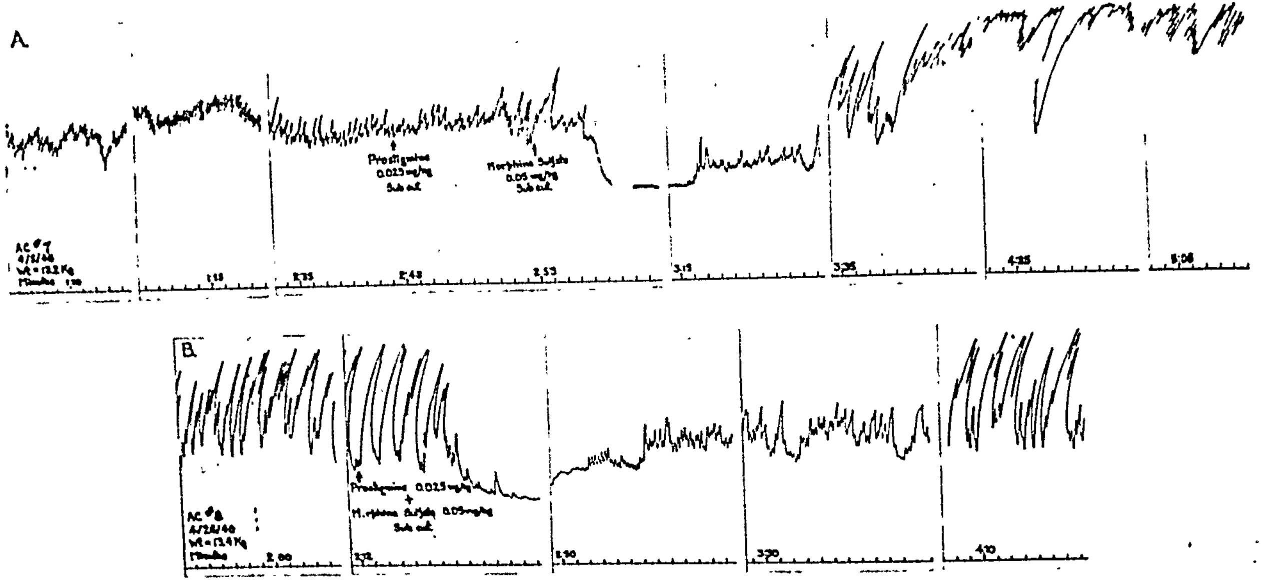


FIG. 3, A. IN THIS TRACING CONSECUTIVE SUBLIMINAL DOSES OF PROSTIGMINE METHYLSULFATE AND MORPHINE WERE GIVEN 10 MINUTES APART

Note typical morphine effects on the stomach followed by marked increase in tonus and amplitude. Time trace, minutes

FIG. 3, B. EFFECTS OF COMBINED SUBLIMINAL DOSES OF MORPHINE AND PROSTIGMINE METHYLSULFATE

Typical morphine effects on the stomach followed with return to normal in about one hour morphine alone. However, as regards the duration of the lowered tonicity, we might add that since 0.05 mgm./kg. of morphine by itself produces no such effect, the effect when combined with prostigmine methylsulfate may be regarded as typical for this combination. At any rate, the small dose of morphine is effectively potentiated by prostigmine.

Prostigmine methylsulfate also causes retention of morphine in the body as we have shown in excretion experiments (unpublished). Such retention might increase the amount of the active form of morphine reaching the loci of action in the body and so render a subliminal dose of morphine effective.

Other signs of potentiation are instances of nausea and vomiting which did not occur when either of the small doses was given alone.

Veach (8) has shown that prostigmine methylsulfate usually causes inhibitory effects on the stomach in humans. As previously indicated he reported that the inhibitory action of atropine on the human stomach was made motor by prostigmine methylsulfate. He (9) further pointed out that the action of morphine consistently caused an increased tonus of that organ. Recently, Hamilton and Curtis (10) have reported that atropine plus prostigmine methylsulfate given as one injection causes motor effects on the human stomach. In the dog we have not been able to obtain any of these effects.

One might argue that the lowering of tonus due to prostigmine methylsulfate was due to the defecation reflex described by Youmans and Meek (11). We feel that the observed effects last far too long to be reflex in nature. On the other hand, we have repeatedly seen marked decreases in gastric tonus due to prostigmine methylsulfate without the occurrence of defecation. Further, to our knowledge, no experiments have shown any relation between the effects of morphine on the stomach and the defecation reflex.

As to the mechanism of the production of these effects, it is difficult to be specific since it has been argued by most workers that the activity of the stomach is dependent upon its previous level of tonus.

We believe that morphine is a cholinergic drug and that prostigmine methylsulfate, a known cholinergic drug, potentiates its action on the stomach. It is difficult to explain why prostigmine methylsulfate or morphine alone cause chiefly inhibition of this viscus. The work of Brücke and Stern (12) indicates that the vagus may contain adrenergically functioning nerves which in this instance would be stimulated by a cholinergic mechanism. Ivy and Gray (13) have pointed out that with a high tonus, stimulation of the vagus always causes inhibition and that even with a low tonus, when atropine has been given previously, the vagus stimulation caused a fall in tonus of the stomach. There is also the possibility of activation of these inhibitory (cholinergic) fibers at the autonomic ganglia where acetylcholine normally is functional in transmission.

We offer no explanation for the marked increase in tonus and rhythmical activity following the morphine-like effect when an inactive dose of morphine is given after an ineffective dose of prostigmine methylsulfate, but it was so consistent in occurrence that we feel it worth reporting.

## SUMMARY

1. In appropriate subcutaneous and intravenous doses prostigmine methylsulfate is inhibitory to the tonus and contractions of the stomach in unanesthetized dogs.

2. When inactive doses of morphine are given following subliminal doses of prostigmine methylsulfate, a typical morphine-like effect is seen in the stomach of unanesthetized dogs. This is followed by a rise in the tonus and by rhythmical activity increased above the normal level.

3. Individually inactive doses of morphine and prostigmine methylsulfate combined in one injection produce a typical morphine-like effect on the stomach.

4. These results indicate that the effect of morphine on the stomach resembles that of a cholinergic drug and that it is potentiated by prostigmine.

5. Using the dog, we cannot confirm the reports of Veach, *et al.*, and of Hamilton and Curtis, that prostigmine methylsulfate reverses the action of atropine in the human stomach.

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# THE PYRETIC ACTION ON RATS OF SMALL DOSES OF MORPHINE

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While investigating the effect on the rat's temperature of non-narcotizing doses of central nervous system depressants combined with exposure to cold, we encountered a phenomenon hitherto unconvincingly described. This is the pyretic action of morphine in sub-hypnotic doses. It was decided to investigate this phenomenon in the rat and, if consistently present, to analyze its mechanism.

A survey of the literature reveals that the pyretic action of morphine has been a controversial subject. Starkenstein (1924) in Heffter's Handbuch der Experimentellen Pharmakologie, states that "old experiments on animals and man have shown that the temperature is raised by small doses of morphine and lowered by toxic doses." The literature was searched for these old experiments. Von Schroff (1868) stated that small doses of morphine (up to 14 mgm.) in man, cause an increase in the pulse rate and in the temperature of the tongue and hand. No figures were given. The experimental work of Gescheidlen (1869) was confined to one small female dog with a subnormal temperature (36.8°C.) which showed a rise of 1°C. twelve hours after the administration of 5 mgm. of morphine acetate. There was no control. From this experiment he concluded that morphine raises the body temperature of dogs. From observations on rabbits Manassein (1871) concluded that medium and large doses of morphine hydrochloride produce a drop in body temperature whereas small doses, $\frac{1}{16}$ to $\frac{1}{4}$ grain, produce at times a rise. The work of Kersch (1871) gives the results of 25 experiments with dogs in which doses of 0.3 to 1.3 grams of morphine acetate were employed. No weights or temperature readings were given. He states that after morphine there is a rise of temperature, increased respiration, narrowing of pupils and excitement followed later by paralysis, convulsions and death. The temperature rise was described when the animal was in the "excitement" stage. Köhler (1876) wrote, "the temperature rises after the administration of small doses (morphine) and later falls." No evidence was cited. Rükert (1882) concluded that morphine, regardless of the dosage, produced no temperature rise in either dogs or rabbits. Contrariwise, with doses so small that they produce no obvious effects, the temperature may fall 3°C, while with larger doses the drop is greater. Working with rabbits, Gottlieb (1890) reduced an experimental brain puncture fever by the use of sub-hypnotic doses of morphine. Normal rabbits placed in a warm box (31 to 32°C.) after the administration of 10 to 20 mgm. of morphine, exhibited no change in body temperature.

In 1922 Stewart and Rogoff described hyperthermia in cats after the administration of small but exciting doses of morphine. Chahovitch and Vichnjitch (1928) found slight temperature elevation in rats after the administration of morphine in doses of approximately 50 milligrams per kilogram. Girndt and

Lipshitz (1931), working with rabbits, found that morphine hydrochloride in doses of 1 to 20 milligrams per kilogram causes a significant drop in temperature. Helfrich (1935) concluded from his researches that morphine sulphate in small doses produces considerable pyrexia in cats and guinea pigs. This reaction was less intense in dogs, mice and rabbits. In 1940 Spragg reported that chimpanzees exhibit a decrease in temperature after the first few of a series of daily doses of morphine (0.1 milligram per kilo), whereas, subsequent administration of the drug produced no temperature change.

From the foregoing it would seem that the evidence for a consistent pyretic action of morphine is inconclusive.

PROCEDURES. Young male, albino rats, weighing from 250 to 300 grams, were given morphine sulphate (0.75% solution) subcutaneously. Colonic temperatures were taken at intervals by means of a mercury thermometer. The animals were allowed water freely during the experiments which were carried out at temperatures of about 25°C. All experiments were started in the morning and completed in the same afternoon.

Metabolic determinations were made on the Haldane metabolism train, in a room held constantly at 28°C., food and water having been removed from the animals twelve hours before the start of the metabolism run.[1] After an initial hour's run, morphine was administered and the temperature was followed at hourly intervals without removing the animal from its chamber. The body temperature determinations were made just before each hour's metabolism run and after the final one.

Adrenalectomy and adreno-demedullation were performed through the lumbar approach under anesthesia by ether or intraperitoneal nembutal. After these procedures the animals were kept in a thermostat-controlled warm room (28°C.). The adrenalectomized animals were divided into three groups, one of which was allowed 0.9% sodium chloride solution, another tap water and the third tap water containing adrenal cortical extract (Upjohn), 1 cc. to 30 cc. of water. Since the cortical extract contained alcohol, control experiments with alcohol were performed. Morphine was administered 24 hours following the operation.

Hypophysectomy through the floor of the sella turcica was performed under ether anesthesia ten days after adreno-demedullation.

RESULTS. Morphine sulphate in doses of 1, 2, 5, 10, 15 and 20 mgm. per kilogram consistently produced a moderate hyperthermia in rats. In most instances the degree of pyrexia elicited by these doses was substantially the same. The fever usually reached its maximum within two hours, then subsided slowly so that within five hours after the administration of the drug the temperature had returned to normal limits. These doses produced a well developed catalepsy and exophthalmos which ran a course concomitant with the temperature. The critical dose was around 30 mgm. per kilogram. With doses of 40 to 90 mgm. per kilogram hyperthermia was absent, and in some instances hypothermia resulted (figure 1). Catalepsy and exophthalmos, however, occurred maximally regardless of the dosage. Tatum has described a mild form of catalepsy in rabbits after the administration of morphine.

Morphine administered to bilaterally adrenalectomized rats maintained on tap water post-operatively did not induce pyrexia or exophthalmos but did produce catalepsy. On the other hand, 20 adrenalectomized animals which were allowed post-operatively tap water containing adrenal cortical extract or 0.9% sodium chloride developed, after morphine, pyrexia, exophthalmos and catalepsy (fig. 2). Since the cortical extract contained alcohol (10%) control experiments were carried out which proved that the alcohol was not a factor in the production of the pyrexia.

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[1] The author is indebted to Dr. M. Puzak for the metabolic determinations.

Two adrenalectomized rats maintained on tap water that received epinephrine (0.1 milligram) ½ hour prior to the administration of morphine failed to develop pyrexia. On the other hand bilateral adreno-demedullation in eleven rats did not prevent the appearance of the morphine temperature rise, catalepsy and exophthalmos. Likewise, hypophysectomy alone or in previously adreno-demedullated rats did not inhibit the morphine-induced pyrexia.

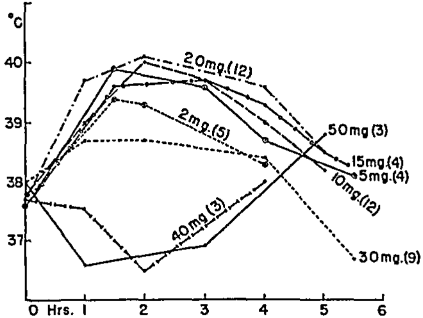

FIG. 1. BODY TEMPERATURE CHANGES AFTER THE ADMINISTRATION OF MORPHINE SULPHATE

In every curve, each point represents the average temperature for the number of rats given in parenthesis. The dosage, in milligrams per kilogram of body weight, is given with its respective curve.

*Metabolic rate in morphine fevered rats.* To determine the changes in heat production resulting from the small doses of morphine which produce fever, rat metabolism studies were made which are presented in tables 1–3. The $CO_2$ output of the individual animals was taken as the safest index of metabolism because it is collected and weighed directly from the animal. However, it was first established as an index of metabolism after small doses of morphine (which do not essentially disturb ventilation) by preliminary experiments in large rats weighing about 350 grams. Such a rat untreated yielded 778 mgm. $CO_2$ per hour, while two rats receiving morphine (10 mgm./kg.) yielded respectively, 723 and 848 mgm. $CO_2$ per hour. In the untreated animal the heat production was .137 calories per gram per 24 hours, while the corresponding calories in the morphine treated rats were respectively, .139 and .146. In all three experiments the ratio between $CO_2$ and calories is so nearly the same that the former figure may be used as a metabolism determination.

There was a 10% increase in metabolic rate associated with the morphine pyrexia induced in a series of rats. Adrenalectomized rats, which exhibited some reduction in metabolic rate, showed, however, no increase following the administration of morphine.

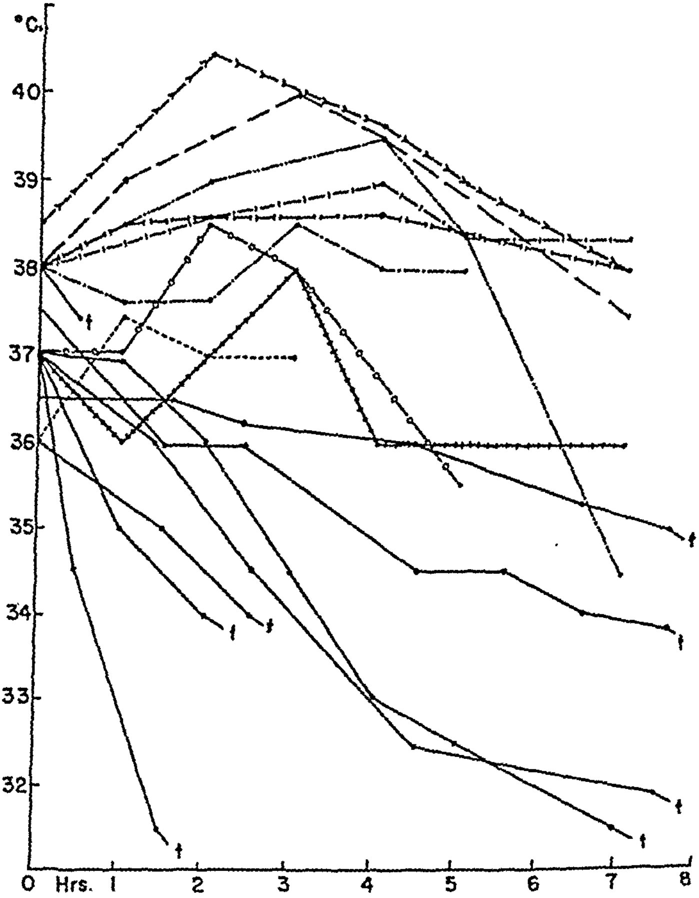

FIG. 2. BODY TEMPERATURE CHANGES IN ADRENALECTOMIZED RATS AFTER THE ADMINISTRATION OF MORPHINE SULPHATE

Each curve represents one experiment. The temperature changes of the rats that received cortical extract are represented by broken lines whereas the temperature changes of those that did not receive cortical extract are represented by solid lines.

The sign (†) signifies that death occurred shortly after the last temperature determination.

The dosage of morphine in each experiment was 10 mgm./kg.

Acute tolerance for morphine was decreased by adrenalectomy, the drug being found lethal in doses which are non-depressant for the intact animal (fig. 2). Contrariwise, after adreno-demedullation or hypophysectomy the rat's tolerance for morphine did not change.

TABLE 1

*Metabolic studies after morphine sulphate in rats weighing 326–360 grams*

| HOURS | METABOLISM, (mgm. $CO_2$/hr.) | | | | TEMPERATURE, °C. | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Av. | A | B | C | Av. |
| * | | | | | 37.5 | 37.0 | 37.8 | 37.4 |
| 0 | 0.646 | 0.642 | 0.590 | 0.626 | 37.5 | 37.2 | 38.0 | 37.5 |
| 1 | 0.543 | 0.627 | 0.672 | 0.610 | 37.5 | 37.5 | 40.0 | 38.3 |
| 2 | 0.708 | 0.694 | 0.661 | 0.688 | 40.0 | 40.5 | 39.8 | 40.1 |
| 3 | | | 0.580 | 0.580 | 39.5 | 39.4 | 39.0 | 39.3 |

* Just before animals were placed in metabolism chamber. Rats received 10 mgm. per kilogram of morphine sulphate hypodermically. In 10 additional determinations in four rats of similar weight range, after morphine, the metabolism varied from 0.528 to 0.723. In tables 1, 2 and 3, body temperature determinations were made just before each hour's metabolism run and after the final one.

TABLE 2

*Metabolic studies in adrenalectomized rats weighing 346–356 grams*

| HOURS | METABOLISM, (mgm $CO_2$/hr) | | | | TEMPERATURE, °C. | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Av | A | B | C | Av. |
| 0 | | | | | 37.5 | 35.0 | 38.5 | 37.0 |
| 1 | 0.517 | 0.428 | 0.544 | 0.499 | 37.5 | 34.8 | 38.8 | 37.0 |
| 2 | 0.493 | 0.459 | 0.508 | 0.486 | 37.5 | 35.0 | 38.1 | 36.8 |
| 3 | 0.448 | 0.434 | 0.498 | 0.460 | 37.5 | 35.2 | 38.5 | 37.0 |
| 4 | | | 0.502 | 0.502 | 37.5 | 35.7 | 37.3 | 36.8 |

TABLE 3

*Metabolic studies in morphinized adrenalectomized rats weighing 360–375 grams*

| HOURS AFTER MORPHINE | METABOLISM, (mgm. $CO_2$/hr.) | | | | TEMPERATURE, °C. | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Av. | A | B | C | Av. |
| * | | | | | 37.5 | 38.0 | 38.0 | 37.8 |
| 0 | 0.447 | 0.511 | 0.549 | 0.502 | 37.5 | 37.8 | 38.2 | 37.8 |
| 1 | 0.431 | 0.520 | 0.579 | 0.510 | 37.3 | 38.0 | 38.3 | 37.8 |
| 2 | 0.470 | 0.523 | 0.524 | 0.505 | 37.7 | 37.5 | 38.6 | 37.9 |
| 3 | 0.455 | 0.504 | 0.504 | 0.487 | 37.5 | 38.5 | 37.7 | 37.9 |
| 4 | | 0.475 | | 0.475 | dead | 38.6 | 37.2 | 37.9 |
| 5 | | | | | | 37.2 | | 37.2 |

* Just before animals were placed in metabolism chamber. Rats received 10 mgm. per kilogram of morphine sulphate hypodermically

DISCUSSION. Pyrexia may result from increased heat production, decreased heat elimination or a combination of both. The morphine fever in cats described by Stewart and Rogoff was associated with considerable motor excitement. However, they concluded that the pyrexia was not entirely dependent upon muscular activity since undrugged animals in a revolving cage performed more muscular exercise with less rise in temperature than those receiving morphine.

With our rats the situation is entirely different. Morphine, even in doses as small as 1 mgm. per kilo, produces a well developed catalepsy which eliminate an increase in heat production from muscular activity as a causative factor i pyrexia. More conclusive evidence, however, that excessive heat production i not significant for the hyperthermia is derived from the metabolic studies. Th metabolic increase with an average temperature rise of 2.6°C. was only 10% which is more than accounted for as a *result* of the temperature rise (Van' Hoff's Law). In fact that 10% increase in metabolic rate accounts for only 20% *of the calories necessary to raise the animals' temperature an average of 2.6°C.* the other 80% must, therefore, come from heat conservation. Chahovitch an Vichnjitch, in the experiments cited above, failed to obtain an increase in th total metabolism in rats after the administration of morphine.

Morphine does not produce pyrexia in adrenalectomized rats. However, i cats Stewart and Rogoff were able to produce morphine pyrexia with the sam facility regardless of the presence or absence of the adrenals. In the intac animal they found that morphine caused an increase in the rate of epinephrin output. This may have been due to excitement. In our rat experiments, although no assays were made of the epinephrine output after the administratio of morphine, the consistent appearance of exophthalmos at the onset of the feve and its persistence during the pyrexia is certainly suggestive of sympatheti stimulation. The exophthalmos, however, is not dependent entirely upon th epinephrine factor since it occurs also after adreno-demedullation.

Morphine hyperthermia occurs after extirpation of the adrenal medulla but not after total adrenalectomy unless either the cortical hormone or sodium chloride is substituted. Therefore it would seem that salt metabolism, which is normally under the influence of the adrenal cortex, plays an important part in morphine pyrexia.

Tatum had demonstrated that a fundamental property of morphine is stimulation, although in chronic morphinism this may be masked at first by central depression. Likewise, Barbour, Porter and Seelye (1939), in metabolic studies on chronic morphinism in dogs, demonstrated the calorigenic property of this drug. Hambourger also, from work with cerebrally decorticated cats, concluded that morphine produced a stimulating effect on the hypothalamus. It may well be that the basic factor in the causation of the morphine hyperthermia is a stimulation of this part of the central nervous system. This is also suggested by the work of Helfrich in which spinal cats failed to develop morphine pyrexia.

In the literature there is a diversity of opinion concerning the effect of bilateral adrenalectomy on the toxicity of morphine. Scott, the McKays, and Tormo believed from their investigations that adrenalectomy in rats increases the toxicity of morphine while Rogoff concluded that it does not. The present investigation adduces further evidence that at ordinary room temperature (25°C.) morphine is distinctly more toxic in adrenalectomized rats unless they are protected by sodium chloride or adrenal cortical hormone. Since the morphine was administered within 24 hours after adrenalectomy when the body temperature was normal and the general condition excellent, the possible objection that one may be dealing with sick animals is invalidated.

## CONCLUSIONS

1. Small doses of morphine sulphate, 1 to 20 mgm. per kilogram, consistently oduce pyrexia in rats. The critical dose is around 30 milligrams per kilo. rger doses, 40 to 90 mgm. per kilo, produce only hypothermia.

2. The pyrexia is associated with catalepsy and exophthalmos.

3. Adrenalectomized rats, which exhibit reduction in metabolic rate, fail to ow metabolic increase after morphine.

4. The morphine fever and its concomitant exophthalmos may be prevented preliminary bilateral adrenalectomy. Adreno-demedullation, alone or in njunction with hypophysectomy, on the other hand, does not prevent the pearance of the morphine fever, catalepsy and exophthalmos.

5. The administration of adrenal cortical extract or 0.9% sodium chloride to renalectomized rats, restores their ability to develop a morphine pyrexia; ntrariwise, epinephrine does not.

6. Increase in metabolic rate associated with the hyperthermia accounts for % of the necessary calories; the balance is therefore derived from heat conser- tion.

7. Bilateral adrenalectomy in rats enhances the toxicity of morphine whereas reno-demedullation or hypophysectomy fails to influence it.

8. A possible explanation for the pyretic action of morphine is, that the drug ting centrally stimulates the sympathetic nervous system, inducing vasomotor anges, a redistribution of body water and of electrolytes with resultant pyrexia.

The author is greatly indebted to Professor H. G. Barbour for his invaluable unsel during the course of this work.

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# IN VITRO AND IN VIVO STUDIES OF GRAMICIDIN, TYROTHRICIN AND TYROCIDINE

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A bactericidal agent obtained from a spore-bearing soil bacillus was described by Dubos (1) and more recently by other investigators (2, 3, 4). The crude extract obtained from this organism is alcohol-soluble, water-insoluble, and is called tyrothricin; it can be fractionated into two substances, gramicidin and tyrocidine (3), the former possessing activity *in vitro* and *in vivo* whereas the latter is primarily active *in vitro*. All three substances are definitely toxic for animals (5, 6) although there appears to be a wide range between the therapeutic and lethal dose as determined by studies in mice. Other toxic effects of these substances have been reported by Heilman and Herrell (7) and Rammelkamp and Weinstein (8). The following communication is concerned with the *in vitro* and *in vivo* activity, the *in vitro* experiments dealing primarily with the effect of serum and blood upon their bactericidal properties.

*Materials and Methods.* Strains of *Streptococcus hemolyticus*, *Staphylococcus aureus*, type I *pneumococcus*, *Clostridium welchii*, *Clostridium tetani*, *Eberthella typhi* and *Escherichia coli* were employed as test organisms. All cultures, with the exception of the strict anaerobes, were grown in brain heart infusion media. For the growth of these organisms the above medium was supplemented with 0.1 per cent agar-agar. Excellent growth was obtained in 6 to 10 hours at 37°C.

The gramicidin, tyrocidine and tyrothricin[1] suspensions were prepared by dissolving the solid preparations in 95 per cent ethyl alcohol and diluting the alcohol solution with sterile distilled water or broth to the desired concentration. Fresh suspensions were made for each experiment.

*In vitro* STUDIES. In the first series of experiments, a modified phenol coefficient technique was used. The gramicidin, tyrocidine and tyrothricin suspensions were mixed with the cultures of bacteria and incubated at 37°C. for periods ranging from 5 minutes to 24 hours. Subtransfers were then made into sterile broth and incubated at 37°C. for 48 hours. The materials were used in concentrations of 1:8000 to 1:512,000 upon cultures of *Cl. welchii*, *Cl. tetani*, type I pneumococcus and *Staph. aureus*. Tyrothricin and tyrocidine proved to be considerably more effective than gramicidin against these cultures (table 1). The particular strain of *Staph. aureus* showed considerable resistance to these agents although other strains of the same organism were found to be more readily affected. Tyrocidine and tyrothricin produced their killing effect within 5 to 10 minutes, whereas gramicidin was essentially without effect even over a 24 hour period. It is to be noted, however, that this method of testing only measures bactericidal activity, and substances such as sulfanilamide, which are primarily bacteriostatic, appear to be without effect under these conditions. This is particularly true when the original inoculum is as large as that used in the phenol coefficient technique.

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[1] The materials used in these experiments were prepared by Dr. Max Tishler from cultures supplied by Dr. J. L. Stokes of the Research Laboratories of Merck & Co. Inc.

Experiments were therefore undertaken to determine the bacteriostatic activity of gramicidin using Kolmer's method (9). A series of dilutions were prepared of the test agent in sterile water, in such a manner that these were 10 times more concentrated than the final dilution desired. To 1 cc. of each dilution, 9 cc. of seeded culture media were added, the latter being prepared by adding 1 cc. of a 10 hour culture to 99 cc. of broth. The cultures were then incubated at 37°C. and subtransfers made into sterile broth at given intervals over a 24 hour period. By examining the cultures containing the active substances as well as the subtransfers after 24 hours incubation, both the bacteriostatic and bactericidal effect could be ascertained.

TABLE 1

*Bactericidal effect of tyrothricin, gramicidin and tyrocidine (modified phenol coefficient technique)*

| SUBSTANCE | TEST ORGANISM | MINIMAL EFFECTIVE BACTERICIDAL CONCENTRATION PERIOD OF CONTACT BETWEEN TEST AGENT AND THE BACTERIA | | | |
|---|---|---|---|---|---|
| | | *5 minutes* | *10 minutes* | *15 minutes* | *24 hours* |
| Tyrothricin | *Diplococcus pneumoniae* type I | 1:512,000 | 1:512,000 | <1:512,000 | <1:512,000 |
| | *Staph. aureus* #2017 | 1:30,000 | 1:40,000 | 1:45,000 | 1:45,000 |
| | *Cl. welchii* | 1:12,000 | 1:16,000 | 1:16,000 | 1:25,000 |
| | *Cl. tetani* | 1:200,000 | 1:400,000 | 1:400,000 | <1:400,000 |
| Gramicidin | *Diplococcus pneumonia* type I | >1:8000 | >1:8000 | >1:8000 | >1:8000 |
| | *Staph. aureus* #2017 | >1:8000 | >1:8000 | >1:8000 | >1:8000 |
| | *Cl. welchii* | >1:8000 | >1:8000 | >1:8000 | >1:8000 |
| | *Cl. tetani* | >1:8000 | >1:8000 | >1:8000 | >1:8000 |
| Tyrocidine | *Diplococcus pneumonia* type I | 1:500,000 | <1:512,000 | <1:512,000 | <1:512,000 |
| | *Staph. aureus* #2017 | 1:25,000 | 1:40,000 | 1:40,000 | 1:40,000 |
| | *Cl. welchii* | 1:50,000 | 1:50,000 | 1:60,000 | <1:75,000 |
| | *Cl. tetani* | 1:100,000 | 1:200,000 | 1:400,000 | 1:400,000 |

Using the foregoing procedure and working with strains of *Cl. welchii*, *Strep. hemolyticus*, type I pneumococcus and *Staph. aureus*, we obtained the results shown in table 2. In general these results are in close agreement with those obtained in the previous experiments. Tyrocidine and tyrothricin were actively bactericidal against all bacteria except *Staph. aureus*, whereas gramicidin showed only a comparatively slight bactericidal action. Gramicidin, however, showed a bacteriostatic effect in spite of the large number of test organisms used in the initial inoculum. If the initial inoculum was such that only 20 to 50 thousand cells were used, gramicidin was much more effective.

Having established that tyrocidine and tyrothricin are bactericidal while gramicidin is primarily bacteriostatic, we thought it important to determine the activity of these substances in the presence of serum. A number of tests were performed using Kolmer's procedure and a 10 per cent serum-broth mixture as the test medium. One cc. of serum and 0.1 cc. of the test substances were mixed for 5 minutes before adding 9 cc. of the seeded culture medium. Using the same cultures and the same concentration employed in the previous experiments we found that serum reduced the bactericidal effect of tyrocidine and tyrothricin considerably, whereas the bacteriostatic action of gramicidin was only inhibited to a slight degree.

TABLE 2

*Bacteriostatic and bactericidal effect of gramicidin, tyrothricin and tyrocidine (Kolmer's technique)*

| SUBSTANCE | TEST ORGANISM | MINIMAL EFFECTIVE BACTERIOSTATIC CONCENTRATION | MINIMAL EFFECTIVE BACTERICIDAL CONCENTRATION PERIOD OF CONTACT BETWEEN TEST AGENT AND THE BACTERIA | | |
|---|---|---|---|---|---|
| | | | *15 minutes* | *30 minutes* | *60 minutes* |
| Tyrothricin | *Streptococcus hemolyticus* | 1:1,000,000 | 1:40,000 | 1:50,000 | 1:50,000 |
| | *Diplococcus pneumoniae* type I | 1:300,000 | 1:40,000 | 1:70,000 | 1:90,000 |
| | *Staph. aureus* S.D. | 1:8000 | >1:8000 | >1:8000 | >1:8000 |
| | *Cl. welchii* | 1:100,000 | >1:8000 | 1:16,000 | 1:16,000 |
| Gramicidin | *Streptococcus hemolyticus* | 1:1,000,000 | 1:16,000 | 1:20,000 | 1:24,000 |
| | *Diplococcus pneumoniae* type I | <1:300,000 | 1:8000 | 1:11,000 | 1:36,000 |
| | *Staph. aureus* S.D. | >1:8000 | >1:8000 | 1:8000 | >1:8000 |
| | *Cl. welchii* | 1:500,000 | >1:8000 | >1:8000 | 1:8000 |
| Tyrocidine | *Streptococcus hemolyticus* | 1:300,000 | 1:100,000 | 1:100,000 | 1:100,000 |
| | *Diplococcus pneumoniae* type I | 1:300,000 | 1:200,000 | 1:200,000 | 1:200,000 |
| | *Staph. aureus* S.D. | <1:8000 | >1:8000 | >1:8000 | >1:8000 |
| | *Cl. welchii* | 1:100,000 | 1:100,000 | 1:100,000 | 1:100,000 |

Following Waksman and Woodruff's (10) procedure the active substances were incorporated into melted agar, which was permitted to solidify. Next, the agar plates were streaked with a variety of gram-positive and gram-negative bacteria and incubated at 37°C. for 24 hours. Plates were examined for the presence or absence of growth. Essentially the same results were obtained as presented in the foregoing section. With the exception of tyrocidine, none of the substances was effective against the gram-negative organisms even in concentrations as high as 800 micrograms per cc. At this concentration tyrocidine appeared to exert a slight inhibitory effect on *E. typhi*, but not on *E. coli*.

ROTATING RACK EXPERIMENTS. A large number of *in vitro* experiments were performed with the method described by Lockwood (11) which resembles as closely as possible conditions *in vivo*. Mouse-virulent strains of the above organisms were grown at 37°C. After 6 hours incubation the cultures were diluted in broth, blood or serum, to $10^{-5}$ and 2 cc. of this culture dilution placed in small test tubes. Under these conditions the cultures of streptococci and pneumococci contained approximately 40 to 80,000 cells, whereas the culture of *Staph. aureus* contained almost twice this number of cells. The desired amount of the test agent contained in 0.1 cc. of broth was then added to each tube, which was now sealed and rotated slowly on a mixing machine at 37°C. At suitable intervals the tubes were opened and, by making blood agar pour plates of 0.1 cc. of the contents, the number of living organisms in each tube was determined.

TABLE 3

*In vitro efficacy of gramicidin, tyrothricin and tyrocidine in broth*
*(Rotating rack technique)*

| TUBE NUMBER | SUBSTANCE | MICROGRAMS PER CC. OF BLOOD | NUMBER OF VIABLE BACTERIA PER 0.001 CC. OF BLOOD | | | | |
|---|---|---|---|---|---|---|---|
| | | | Time in hours | | | | |
| | | | 0 | 2 | 4 | 10 | 24 |
| 1 | Gramicidin | 128 | 38 | 4 | 0 | 0 | 0 |
| 2 | | 64 | 44 | 20 | 8 | 0 | 0 |
| 3 | | 32 | 42 | 30 | 16 | 14 | 15 |
| 4 | | 16 | 46 | 38 | 32 | 30 | 40 |
| 5 | | 8 | 38 | 34 | 48 | 40 | ∞ |
| 6 | | 4 | 42 | 38 | 38 | 80 | ∞ |
| 7 | | 2 | 40 | 38 | 39 | 75 | ∞ |
| 8 | | 1 | 38 | 44 | 36 | 82 | ∞ |
| 9 | Tyrothricin | 16 | 44 | 0 | 0 | 0 | 0 |
| 10 | | 8 | 44 | 0 | 0 | 0 | 0 |
| 11 | | 4 | 48 | 0 | 0 | 0 | 0 |
| 12 | | 2 | 40 | 38 | 20 | 5 | 0 |
| 13 | | 1 | 39 | 40 | 42 | 80 | ∞ |
| 14 | Tyrocidine | 16 | 38 | 0 | 0 | 0 | 0 |
| 15 | | 8 | 42 | 0 | 0 | 0 | 0 |
| 16 | | 4 | 42 | 2 | 0 | 0 | 0 |
| 17 | | 2 | 41 | 0 | 0 | 0 | 0 |
| 18 | | 1 | 45 | 48 | 82 | ∞ | ∞ |
| 19 | Controls | 0 | 42 | 4200 | ∞ | ∞ | ∞ |
| 20 | | 0 | 37 | 6100 | ∞ | ∞ | ∞ |

Quantities of gramicidin, tyrocidine and tyrothricin ranging from 1 to 256 micrograms per cc. of the test medium were used in these experiments. Since the results obtained with *Strep. hemolyticus*, type I pneumococcus or a non-resistant strain of *Staph. aureus* were of the same order, only the data dealing with the *Strep. hemolyticus* are presented.

When brain-heart infusion medium was employed without the presence of serum or blood, tyrocidine and tyrothricin were actively bactericidal to streptococci; a concentration of 4 to 8 micrograms per cc. of either agent producing complete sterilization within 1 to 2 hours (table 3). In similar concentrations, the action of gramicidin appeared to be primarily bacteriostatic; it exerted a bactericidal action only in much higher concentrations.

TABLE 4

*In vitro efficacy of gramicidin, tyrothricin and tyrocidine in blood*
*(Rotating rack technique)*

| TUBE NUMBER | SUBSTANCE | MICROGRAMS PER CC. OF BLOOD | NUMBER OF VIABLE BACTERIA PER 0.001 CC. OF BLOOD | | | | |
|---|---|---|---|---|---|---|---|
| | | | Time in hours | | | | |
| | | | *0* | *2* | *4* | *10* | *24* |
| 1 | *Gramicidin* | *128* | *82* | *36* | *44* | *0* | *0* |
| 2 | | 64 | 55 | 41 | 42 | 88 | ∞ |
| 3 | | 32 | 70 | 56 | 79 | 987 | ∞ |
| 4 | | 16 | 68 | 53 | 360 | 3800 | ∞ |
| 5 | | 8 | 65 | 94 | 975 | 4200 | ∞ |
| 6 | | 4 | 81 | 124 | 1045 | ∞ | ∞ |
| 7 | | 2 | 55 | 256 | 2090 | ∞ | ∞ |
| 8 | | 1 | 58 | 410 | 2345 | ∞ | ∞ |
| 9 | Tyrothricin | 128 | 63 | 1 | 0 | 0 | 0 |
| 10 | | 64 | 72 | 1 | 0 | 0 | 0 |
| 11 | | 32 | 58 | 34 | 30 | 750 | ∞ |
| 12 | | 16 | 60 | 74 | 108 | 2900 | ∞ |
| 13 | | 8 | 73 | 63 | 505 | 4700 | ∞ |
| 14 | | 4 | 70 | 171 | 1060 | ∞ | ∞ |
| 15 | | 2 | 64 | 393 | 2402 | ∞ | ∞ |
| 16 | | 1 | 68 | 507 | 4440 | ∞ | ∞ |
| 17 | Tyrocidine | 128 | 64 | 0 | 0 | 0 | 0 |
| 18 | | 64 | 71 | 17 | 11 | ∞ | ∞ |
| 19 | | 32 | 78 | 875 | ∞ | ∞ | ∞ |
| 20 | | 16 | 60 | 597 | ∞ | ∞ | ∞ |
| 21 | | 8 | 54 | 940 | ∞ | ∞ | ∞ |
| 22 | | 4 | 70 | 1020 | ∞ | ∞ | ∞ |
| 23 | Control | 0 | 64 | 1170 | ∞ | ∞ | ∞ |
| 24 | | 0 | 71 | 1045 | ∞ | ∞ | ∞ |
| 25 | | 0 | 57 | 1084 | ∞ | ∞ | ∞ |

When blood or serum was used as the test medium, the bactericidal action of tyrocidine was markedly reduced. Under these conditions concentrations of 128 micrograms of tyrocidine per cc. of blood were required to produce the same killing effect as 2 micrograms in brain-heart infusion broth. On the other hand, blood or serum had considerably less effect on the action of gramicidin, as shown in table 4. Tyrothricin, like tyrocidine, lost its bactericidal properties in blood or serum and, like gramicidin, produced only a bacteriostatic effect in the weaker concentrations. It would seem therefore that the bactericidal action of the tyrocidine portion of tyrothricin is inhibited by blood and the bacteriostatic action of the gramicidin portion may then manifest itself. It will be noted that the bacteriostatic action of gramicidin and tyrothricin in blood was most striking during the first 4 hours of incubation and that eventually almost all the cultures made abundant growth. This apparent lack of bacteriostatic activity after a 4 hour incubation period may be due to a toxic effect of gramicidin on the phagocytes since at this time there is direct evidence of the destruction of red cells, as shown by hemolysis.

When blood agar plates were made from cultures which were sterilized by gramicidin, small light zones, not typical zones of hemolysis as a result of the presence of viable streptococci, could be observed. Subcultures of these areas into sterile broth did not produce growth. Furthermore, quantities of gramicidin similar to those employed in the test did not produce these effects when added to blood agar plates. The nature of these zones was not investigated further.

*In vivo* STUDIES. Swiss mice were injected intraperitoneally with a killing dose of a virulent test culture grown under conditions described in the *in vitro* experiments. Immediately after the injection of the bacteria the animals were treated with the various preparations by intraperitoneal administration, thus permitting direct mixing of the bacteria and the test substance in the peritoneal cavity of the mice. Additional experiments were performed in which mice infected by the intraperitoneal method were treated by oral, subcutaneous and intravenous routes. Both single and multiple dosing were employed in these experiments. Infections with *Staph. aureus* and *Cl. welchii* were produced with and without the aid of 4 per cent gastric mucin.

Single injections of gramicidin or tyrothricin in doses of 8 to 16 micrograms per mouse afforded protection to mice infected with 10,000 lethal doses of streptococci or pneumococci when both the bacteria and test agent were given intraperitoneally (table 5). With smaller doses of gramicidin or tyrothricin, the lives of mice were prolonged for 3 to 4 days although eventually most of these animals died. Under the same conditions tyrocidine was essentially without effect even in much larger doses. It is of interest to note the close agreement between the effective *in vivo* dose and those obtained *in vitro* using blood as the test medium. Smears prepared from the peritoneal fluids of mice treated with gramicidin or tyrothricin indicated phagocytosis of the infecting organisms. Mice infected with *Staph. aureus*, or wash *Cl. welchii* cells and treated with 4 to 32 micrograms of gramicidin or tyrothricin survived longer than the controls, but eventually all the animals died. The results of the experiments dealing with staphylococcal and *Cl. welchii* infections were complicated by the fact that unless the bacteria were administered in 4% mucin, too many bacteria had to be given in order to produce a fatal infection in the untreated control mice. When a large number of bacteria were used as the inoculum in the *in vitro* experiments gramicidin was not very effective even though it showed considerable activity against the same strain when a smaller number of cells were used. On the other hand, when 4% gastric mucin was used, which permitted the use of dilutions of $10^{-4}$ or more, the mucin appeared to interfere with the action of gramicidin or tyrothricin, since the treated animals died almost as rapidly as the untreated controls in spite of large doses of the test agents. This antagonistic action of mucin was also found in streptotoccal and pneumococcal infections and may possibly be due to the interfering effect of mucin upon phagocytosis.

TABLE 5

*Efficacy of gramicidin, tyrothricin and tyrocidine in streptococcal infections in mice*

| | |
|---|---|
| Infection | *Streptococcus hemolyticus* #1685 |
| Age of culture | 6 hours |
| Infection | 0.5 cc. $10^{-4}$ dilution in broth |
| Treatment | Single dose intraperitoneally immediately after the bacterial inoculation |

| SUBSTANCE | MICROGRAMS PER MOUSE | NO. OF MICE | NUMBER MICE SURVIVING AFTER DAYS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Gramicidin | 2 | 50 | 50 | 40 | 20 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 50 | 50 | 38 | 20 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| | 8 | 50 | 50 | 50 | 42 | 41 | 35 | 35 | 35 | 35 | 35 | 35 |
| | 16 | 50 | 50 | 50 | 46 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| | 32 | 50 | 50 | 49 | 48 | 45 | 45 | 45 | 45 | 45 | 45 | 45 |
| Tyrothricin | 2 | 50 | 50 | 48 | 24 | 12 | 2 | 2 | 2 | 2 | 2 | 2 |
| | 4 | 50 | 50 | 47 | 27 | 14 | 14 | 14 | 14 | 14 | 14 | 14 |
| | 8 | 50 | 50 | 46 | 40 | 40 | 37 | 37 | 37 | 37 | 37 | 37 |
| | 16 | 50 | 50 | 44 | 42 | 42 | 42 | 42 | 42 | 42 | 42 | 42 |
| | 32 | 50 | 50 | 50 | 47 | 44 | 44 | 44 | 44 | 44 | 44 | 44 |
| Tyrocidine | 2 | 50 | 4 | 0 | 0 | 0 | | | | | | |
| | 4 | 50 | 40 | 0 | 0 | 0 | | | | | | |
| | 8 | 50 | 40 | 0 | 0 | 0 | | | | | | |
| | 16 | 50 | 42 | 0 | 0 | 0 | | | | | | |
| | 32 | 50 | 40 | 3 | 0 | 0 | | | | | | |
| | 64 | 50 | 38 | 0 | 0 | 0 | | | | | | |
| | CULTURE DILUTION | | | | | | | | | | | |
| Controls | $10^{-4}$ | 50 | 0 | 0 | 0 | | | | | | | |
| | $10^{-5}$ | 25 | 0 | 0 | 0 | | | | | | | |
| | $10^{-6}$ | 25 | 4 | 0 | 0 | | | | | | | |
| | $10^{-7}$ | 25 | 12 | 0 | 0 | | | | | | | |
| | $10^{-8}$ | 25 | 13 | 1 | 0 | | | | | | | |

When animals were infected by the intraperitoneal route and treated subcutaneously, orally, or by vein at frequent intervals, little or no protection was afforded by gramicidin, tyrothricin or tyrocidine. Further, if mice were infected intravenously and treated by intraperitoneal injection of 4, 8, 16 or 32 micrograms of the test agents no protective effect was found. It would seem therefore that gramicidin and tyrothricin are only effective when placed in direct contact with the bacteria. Additional evidence in support of this view was obtained by increasing the interval between injection and treatment for varying lengths of time. A group of mice were injected intraperitoneally with 100 to 1000 lethal doses of pneumococci and treated intraperitoneally ½ to 6 hours after inoculation. Some of the mice treated immediately after the infecting dose served as controls. When therapy was delayed for 30 minutes some protective effect was observed, but all the mice died within 2–3 days. If treatment was delayed for 2 hours or more, no evidence of protection was found.

In the foregoing experiments tyrocidine showed little or no activity *in vivo.* Tyrocidine, however, was found to afford excellent protection to mice in doses of 16 to 32 micrograms if it was allowed to mix *in vitro* with the infecting bacteria for 1 to 5 seconds prior to animal inoculation. Although the latter fact has little practical significance, it does indicate the rapidity with which tyrocidine acts in the absence of body fluids.

Discussion. From the work presented it would seem that gramicidin in small quantities has a bacteriostatic and limited bactericidal action on gram-positive bacteria *in vitro* and that a similar bacteriostatic action occurs *in vivo*, which is augmented by phagocytosis. This bacteriostatic action would appear to prevent the multiplication of the organisms at their usual rate, thus protecting the phagocytes during the stage of their accumulation. In this respect gramicidin is similar to sulfanilamide. Tyrothricin exerts in blood a bacteriostatic effect similar to that of gramicidin, apparently by virtue of its gramicidin content, whereas tyrocidine is essentially without action in the presence of blood or serum under the same conditions. In broth, however, small quantities of tyrocidine and tyrothricin are actively bactericidal, whereas high concentrations of gramicidin are required to produce the same effect. From the *in vivo* studies it is apparent that gramicidin and tyrothricin afford protection to mice infected with *Strep. hemolyticus* and *Diplococcus pneumoniae*, provided that the agents are given shortly after the inoculation of the infecting bacteria and in the direct location of the bacterial inoculation. Gramicidin and tyrothricin were not found effective when tried under a variety of other experimental conditions.

Since gramicidin is apparently only effective under the above conditions, and since it is definitely toxic when given parenterally to animals (6), its use would seem to be limited to those localized infections which permit treatment by topical application. Studies now in progress suggest that tyrothricin can favorably influence the course of experimental local wounds caused by *Staph. aureus* and *Cl welchii.* Under these conditions tyrothricin can be applied locally in high concentrations without evidence of toxicity. Clinical studies also indicate that tyrothricin may be of definite value in local infections caused by gram positive bacteria.

From the results obtained *in vitro* one might expect a definite protective effect *in vivo* with large doses of tyrocidine. Thus, while tyrocidine sterilized a blood medium in concentrations of 128 micrograms per 1 cc. of blood, similar concentrations *in vivo* afforded essentially no protection to mice. In a previous report (6) tyrocidine has been shown to produce a toxic effect on isolated organs in concentrations of 64 to 128 micrograms per 1 cc. This would suggest, among other things, that large doses of tyrocidine exert a toxic effect on the host, thus in some manner lowering its resistance to infection. The possible toxicity, plus the reduction in activity by body fluids would appear to account for the lack of *in vivo* activity of this substance.

Both the *in vitro* and *in vivo* experiments indicate that tyrothricin is about as active as gramicidin. Since tyrothricin contains only 15 to 20 per cent of gramicidin, which is the active principle *in vivo*, the results suggest a possible synergistic action between tyrocidine and gramicidin or the presence of additional factors in tyrothricin which also possess activity *in vivo*. It was observed that tyrocidine appears to increase the solubility of gramicidin in water. Whether this is an actual increase in solubility or only a reduction in the particle size of gramicidin, thus permitting a greater dispersion of the latter, is not known. This factor might influence the activity of gramicidin sufficiently to account in part for the *in vivo* activity of tyrothricin.

## SUMMARY

1. Tyrocidine and tyrothricin appear to exert a marked bactericidal action on aerobic and anaerobic gram-positive bacteria in the absence of blood or serum. Under similar conditions gramicidin appears to be primarily bacteriostatic.

2. In the presence of blood or serum tyrocidine and tyrothricin lose their bactericidal properties whereas gramicidin retains its bacteriostatic properties. Under these conditions tyrothricin becomes primarily bacteriostatic, apparently by virtue of its gramicidin content.

3. *In vivo* results indicate that when mice are infected and treated by intraperitoneal injection, gramicidin and tyrothricin are active whereas tyrocidine is not. Gramicidin or tyrothricin are only effective when given in direct contact with the infecting bacteria.

4. No protection was afforded to mice infected by intraperitoneal injection when treatment was given by oral, subcutaneous or intravenous administration. Likewise, mice infected by vein were not protected by intraperitoneal treatment.

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# THE PLASMA-CELL PARTITION OF BLOOD LEAD

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Any attempt to understand the mechanisms of lead metabolism must include a study of the way in which the metal is carried in the blood. A fundamental factor of this transport is the quantitative partition of the lead between the plasma and the cells, or the serum and clot. Various opinions concerning this partition are: (*1*) that the lead is present almost entirely in the cell fraction, (*2*) that it is about equally distributed between the blood fractions, and (*3*) that the metal is found principally in the plasma. The first viewpoint is represented by Millon (1), Schmidt (5), Schmidt, Seiser and Litzner (8), Schmidt and Barth (8), Behrens (10), Weyrauch (11), Blumberg and Scott (13), Willoughby and Wilkins (15), and Kehoe, Cholak, and Story (16). Proponents of the second theory include Behrens and Pachur (7), Teisinger (12), Schmitt and Taeger (14), and Tompsett and Anderson (17); while Aub, Fairhall, Minot, and Reznikoff (6) are among those who believe that most of the lead is found in the plasma. Millon (1) stated that normal lead was combined with the blood globulin, while Cozzi (2) claimed that in plumbism the blood lead was combined with the albumin. Riva (3) and Erlenmeyer (4) reported that in experimental lead poisoning the lead was present in the globulin fraction of the proteins. Other concepts have been advanced by Smith, Rathmell, and their coworkers (18, 19, 20), and by Bischoff and coworkers (9).

These conflicting conclusions have resulted largely from differences in the sensitivity and accuracy of the analytical methods employed, and also from analytical variations due to contamination. In the work to be reported in this paper a photometric dithizone method (21) of proved reliability was used. Results obtained by this procedure have been compared with those obtained by a spectrographic (22) and a polarographic (23) method, showing that the procedure possesses satisfactory accuracy and specificity. The need for an extremely precise method, together with the almost complete elimination of contamination, becomes apparent when it is pointed out that a difference of less than one microgram in the quantity of lead found in the blood plasma can mean a difference of over 20% in the proportion of the total lead found in that fraction.

Constant use of the dithizone method cited has indicated that the lead determination itself, exclusive of the sample preparation, is precise to 0.2 microgram. However, lead contamination may occur while the sample is being dried and ignited, so that with quantities of lead below 10 micrograms in the original sample the total error may be as great as 0.5 microgram.

Experimental Methods. Lead acetate was administered to adult female rabbits by spraying a solution of the salt on pellets of a prepared rabbit food. Sufficient lead acetate was used so that each rabbit received about 150 mgm. of lead daily. After eleven days, a blood sample showed 0.04 mgm. of lead per 100 grams of blood; after another week the lead content was 0.06–0.08 mgm. per 100 grams, and it did not rise significantly above this level so long as the same amount of lead acetate was used (three months). After that period the lead in the food was increased to 250 mgm. per day per rabbit for one month, then further increased to 500 mgm. per day. However, the rabbits did not eat this highly contaminated food well and began to lose weight. Therefore, after two weeks the lead content was decreased so that each animal again ingested about 250 mgm. of lead daily. The blood lead level showed a significant rise in each animal during this time.

Previous studies on the partition of lead in blood have been made on serum and clot, or on plasma and cells with the use of anticoagulants, except in one instance when Blumberg and Scott (13) used a sample of hemophilic blood. No record could be found of a series of lead analyses on plasma and cells in which no anticoagulant was used, nor of any comparison of the lead partition in arterial blood with that in venous blood. For that reason arterial and venous blood samples were obtained for lead analysis and separated into plasma and cells, both with and without the use of anticoagulants.

The technic of obtaining blood samples from the rabbits was as follows. A stainless steel needle with a stainless steel hub (not the usual brass hub) was used, with a clean Pyrex hypodermic syringe from which the metal spring clip or plunger guide had been removed. Ten cc., 20 cc., and 30 cc. syringes were used dependent upon the quantity of sample desired. About one cc. of lead-free, heavy mineral oil was put into the dry syringe and some of the oil was expelled through the needle. The blood sample was taken from the left or right heart, depending upon whether arterial or venous blood was desired. If an anticoagulant was used the blood was expelled into a centrifuge tube containing the dry anticoagulant; it was mixed by agitation and centrifuged for five to ten minutes. If no anticoagulant was used the blood was poured (with as little disturbance as possible) into a chilled paraffined centrifuge tube (using lead-free paraffin wax) and cooled in an ice bath for one minute. It was then centrifuged for five to ten minutes.

The plasma was removed as completely as possible by a pipette, expelled into a dry tared silica dish, and the cells (with the small quantity of plasma remaining) were poured into another tared dish. Both dishes were weighed and the samples were then ready for the usual preparation (22) and lead determination (21). All tubes, pipettes, dishes, etc. were cleaned with 50% nitric acid and double distilled water and every possible precaution was taken to avoid lead contamination from dust or other sources.

Lead was added to heparinized blood in some experiments by using a dilute solution of lead chloride; the volume of solution introduced was purposely kept low (about 0.1 cc. per 10 cc. of blood) in order to limit alteration of the water content of the blood and to prevent hemolysis. Lead chloride was used instead of the nitrate or acetate to avoid addition of a foreign ion to the blood The blood was centrifuged about 15 minutes after the lead solution was added.

Human blood from a subject who had been exposed to lead but whose blood lead level had dropped to a normal value, and from another subject whose severe lead exposure had been recently terminated, was analyzed in the same way as the rabbit blood. In this case the sample was drawn from an arm vein with an all stainless steel needle and a 30 cc. oiled Pyrex syringe.

EXPERIMENTAL RESULTS. All results reported in the tables were calculated in the following way: the blood was assumed to be half plasma and half cells by weight, and the quantity of lead in the total plasma (including the small amount left with the cells after the greater part of the plasma had been removed) was calculated and subtracted from the total lead in the sample in order to obtain the quantity actually in the cell fraction.

Table 1 indicates that in rabbits there is no significant difference between the plasma-cell lead partition in the arterial blood and that in the venous blood. At least 90% of the total blood lead is found in the cell fraction; it is not inconceivable that even the small quantity of lead found in the plasma, especially at lower lead levels, is the result of contamination which is unavoidable in the present state of analytical technic.

Tables 2, 3, and 4 show that anticoagulants may be used in the plasma-cell separation of rabbit blood, at least within the limits of the lead concentrations investigated, without causing a significant change in the lead partition. Heparin (about 1 mg. per 10 grams of blood), sodium citrate (0.3 cc. of a solution containing 30 grams in 100 cc., deleaded with dithizone, for each 10 gram sample of blood) and potassium oxalate (about 10 mgm. per 10 grams of blood) were used in these experiments.

TABLE 1

*Lead partition in arterial and venous blood of rabbits*

| ANIMAL IDENTIFICATION | SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|---|
| | | *grams* | *micrograms* | *mg./100 grams* | *micrograms* | *micrograms* | |
| 1 | Arterial | 10.0 | 4.8 | 0.05− | 0.6 | 4.2 | 12.5 |
| 1 | Venous | 10.3 | 4.0 | 0.04− | 0.3 | 3.7 | 7.5 |
| 2 | Arterial | 10.9 | 7.7 | 0.07+ | 0.4 | 7.3 | 5 |
| 2 | Venous | 5.0 | 4.1 | 0.08 | 0.4 | 3.7 | 10 |
| 2 | Arterial | 9.8 | 7.3 | 0.075 | 0.2 | 7.1 | 3− |
| 2 | Venous | 10.0 | 8.5 | 0.085 | <0.1 | 8.5 | <1 |
| 4 | Arterial | 9.3 | 7.0 | 0.075 | 0.1 | 6.9 | 1.5 |
| 4 | Venous | 9.3 | 6.0 | 0.065 | 0.4 | 5.6 | 7 |
| 5 | Arterial | 10.7 | 11.1 | 0.11− | 0.8 | 10.3 | 7 |
| 5 | Venous | 9.7 | 11.2 | 0.11+ | 1.7 | 9.5 | 15 |
| 6 | Arterial | 11 | 8.5 | 0.08− | 0.7 | 7.8 | 8 |
| 6 | Venous | 9.7 | 8.1 | 0.085− | 0.5 | 7.6 | 6 |

Even when the blood lead level was raised by increasing the quantity of lead in the rabbit food, the lead found in the plasma still remained below 10% of that in the whole blood, as shown in table 5. The fact that no significant difference was found between the plasma-cell and the serum-clot partitions of blood lead (table 6) indicates that all these results can probably be compared to those obtained by workers using the serum-clot separation.

The results in table 7 on the plasma-cell lead partition in human blood show that practically all of the lead is in the cell fraction and that heparin has no significant effect upon the partition. Unfortunately, only one sample of human

TABLE 2

*Effect of anticoagulants upon plasma-cell lead partition of rabbit blood treated with heparin*

| ANIMAL IDENTIFICATION | SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|---|
| | | *grams* | *micrograms* | *mg /100 grams* | *micrograms* | *micrograms* | |
| 3 | No anticoagulant | 10.6 | 6.6 | 0.06+ | 0.3 | 6.3 | 5 |
| 3 | With heparin | 8.7 | 5.7 | 0.065 | <0.1 | 5.7 | <2 |
| 4 | No anticoagulant | 9.8 | 8.6 | 0.09− | 0.9 | 7.7 | 10 |
| 4 | With heparin | 9.1 | 7.6 | 0.085 | 0.6 | 7.0 | 8 |
| 5 | No anticoagulant | 10.3 | 7.5 | 0.07+ | 0.35 | 7.15 | 5 |
| 5 | With heparin | 9.0 | 6.6 | 0.075− | 0.3 | 6.3 | 5 |
| 6 | No anticoagulant | 10.3 | 8.5 | 0.08+ | 0.6 | 7.9 | 7 |
| 6 | With heparin | 9.0 | 7.2 | 0.08 | 0.8 | 6.4 | 11 |
| 7 | No anticoagulant | 8.5 | 4.7 | 0.055 | <0.1 | 4.7 | <2 |
| 7 | With heparin | 9.0 | 5.1 | 0.055 | <0.1 | 5.1 | <2 |

TABLE 3

*Effect of anticoagulants upon plasma-cell lead partition of rabbit blood treated with sodium citrate*

| ANIMAL IDENTIFICATION | SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|---|
| | | *grams* | *micrograms* | *mg /100 grams* | *micrograms* | *micrograms* | |
| 2 | No anticoagulant | 9.2 | 5.7 | 0.06+ | 0.3 | 5.4 | 5 |
| 2 | With citrate | 8.9 | 7.5 | 0.085 | 0.7 | 6.8 | 9 |
| 2 | No anticoagulant | 10.8 | 6.2 | 0.06− | 0.5 | 5.7 | 8 |
| 2 | With citrate | 8.6 | 6.1 | 0.07 | 0.7 | 5.4 | 11 |
| 3 | No anticoagulant | 9.9 | 7.0 | 0.07+ | 0.3 | 6.7 | 4 |
| 3 | With citrate | 7.7 | 4.9 | 0.065 | 0.2 | 4.7 | 4 |
| 4 | No anticoagulant | 10.3 | 9.0 | 0.085 | 0.3 | 8.7 | 3.5 |
| 4 | With citrate | 8.2 | 6.9 | 0.085 | 0.5 | 6.4 | 7 |
| 4 | No anticoagulant | 10.0 | 8.4 | 0.085 | 0.4 | 8.0 | 5 |
| 4 | With citrate | 8.8 | 7.2 | 0.08+ | 0.4 | 6.8 | 6 |
| 7 | No anticoagulant | 10.0 | 6.7 | 0.065 | 0.1 | 6.6 | 1.5 |
| 7 | With citrate | 9.1 | 5.7 | 0.065 | <0.1 | 5.7 | <2 |

blood containing larger quantities of lead was available while these observations were in progress.

TABLE 4

*Effect of anticoagulants upon plasma-cell lead partition of rabbit blood treated with potassium oxalate*

| ANIMAL IDENTIFICATION | SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|---|
| | | *grams* | *micrograms* | *mg./100 g.* | *micrograms* | *micrograms* | |
| 3 | No anticoagulant | 10.1 | 6.2 | 0.06+ | 0.3 | 5.9 | 5 |
| 3 | With oxalate | 9.4 | 5.9 | 0.065 | 0.1 | 5.8 | 2 |
| 4 | No anticoagulant | 10.4 | 8.4 | 0.08+ | 0.9 | 7.5 | 11 |
| 4 | With oxalate | 11.0 | 8.6 | 0.08− | 0.1 | 8.5 | 1 |
| 7 | No anticoagulant | 10.0 | 6.7 | 0.065 | <0.1 | 6.7 | <2 |
| 7 | With oxalate | 8.7 | 5.5 | 0.065 | <0.1 | 5.5 | <2 |
| 8 | No anticoagulant | 9.9 | 4.6 | 0.045 | <0.1 | 4.5 | <2 |
| 8 | With oxalate | 9.1 | 3.8 | 0.04+ | <0.1 | 3.8 | <2 |
| 8 | No anticoagulant | 8.5 | 3.5 | 0.04 | <0.1 | 3.5 | <3 |
| 8 | With oxalate | 6.6 | 2.9 | 0.045 | 0.1 | 2.8 | 3 |

TABLE 5

*Plasma-cell partition of blood lead of rabbits—higher lead levels*

| ANIMAL IDENTIFICATION | SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|---|
| Lead in food—approximately 250 mgm./rabbit/day | | | | | | | |
| | | *grams* | *micrograms* | *mgm./100 grams* | *micrograms* | *micrograms* | |
| 2 | No anticoagulant | 13.0 | 12.8 | 0.10 | 0.9 | 11.9 | 7 |
| 4 | No anticoagulant | 12.0 | 10.3 | 0.085 | 0.3 | 10 | 3 |
| Lead in food increased to approximately 500 mgm./day | | | | | | | |
| 2 | Heparin | 12.2 | 14.7 | 0.12 | 2.2 | 12.5 | 15 |
| 2 | No anticoagulant | 9.5 | 13.5 | 0.14 | 1.0 | 12.5 | 7.5 |
| 3 | No anticoagulant | 11.1 | 11.1 | 0.10 | 1.2 | 9.9 | 11 |
| 4 | No anticoagulant | 11.9 | 16.6 | 0.14 | 1.6 | 15 | 10 |
| 7 | No anticoagulant | 11.0 | 32.3 | 0.29 | 1.4 | 30.9 | 4 |
| Lead in food decreased to approximately 250 mgm./day | | | | | | | |
| 2 | No anticoagulant | 10.5 | 15 | 0.14 | <0.1 | 15 | <1 |
| 2 | No anticoagulant | 10.5 | 14.7 | 0.14 | 0.7 | 14 | 5 |
| 3 | No anticoagulant | 10.2 | 7.4 | 0.07+ | 0.4 | 7 | 5 |
| 7 | No anticoagulant | 10.9 | 31.3 | 0.29 | 1.3 | 30 | 4 |
| 8 | No anticoagulant | 11.1 | 10.5 | 0.095 | 0.5 | 10 | 5 |
| 8 | No anticoagulant | 10.7 | 8.1 | 0.075 | 0.6 | 7.5 | 8 |

TABLE 6

*Comparison of plasma-cell and serum-clot lead partitions of rabbit blood*

| ANIMAL IDENTIFICATION | SAMPLE (NO *ANTICOAGULANTS USED*) | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA OR SERUM | LEAD IN CELL FRACTION OR CLOT | PER CENT OF TOTAL LEAD *IN PLASMA* OR SERUM |
|---|---|---|---|---|---|---|---|
| | | *grams* | *micrograms* | *mgm /100 grams* | *micrograms* | *micrograms* | |
| 2 | Plasma-cell | 10.5 | 15 | 0.14 | <0.1 | 15 | <1 |
| | Serum-clot | 10.1 | 14.5 | 0.14 | 0.5 | 14 | 3 |
| 2 | Plasma-cell | 10.5 | 14.7 | 0.14 | 0.7 | 14 | 5 |
| | Serum-clot | 11.3 | 14.2 | 0.13 | 0.7 | 13.5 | 5 |
| 3 | Plasma-cell | 10.2 | 7.4 | 0.07+ | 0.4 | 7.0 | 5 |
| | Serum-clot | 10.7 | 7.3 | 0.07− | 0.4 | 6.9 | 6 |
| 7 | Plasma-cell | 11.0 | 32.3 | 0.29 | 1.4 | 30.9 | 4 |
| | Serum-clot | 10.6 | 31.4 | 0.30 | 1.9 | 29.5 | 6 |
| 7 | Plasma-cell | 10.9 | 31.3 | 0.29 | 1.3 | 30 | 4 |
| | Serum-clot | 10.4 | 30.0 | 0.29 | 1.0 | 29 | 3 |
| 8 | Plasma-cell | 11.1 | 10.5 | 0.095 | 0.5 | 10 | 5 |
| | Serum-clot | 10.8 | 10.3 | 0.095 | 0.3 | 10 | 3 |
| 8 | Plasma-cell | 10.7 | 8.1 | 0.075 | 0.6 | 7.5 | 8 |
| | Serum-clot | 11.7 | 8.9 | 0.075 | 0.9 | 8.0 | 10 |

TABLE 7

*Plasma-cell partition of lead in human blood*

| SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|
| | *grams* | *micrograms* | *mgm /100 grams* | *micrograms* | *micrograms* | |
| No anticoagulant | 9.5 | 3.5 | 0.035+ | 0.2 | 3.3 | 6 |
| With heparin | 10.0 | 3.5 | 0.035 | 0.1 | 3.4 | 3 |
| No anticoagulant | 10.6 | 3.4 | 0.03+ | <0.1 | 3.4 | <3 |
| With heparin | 10.3 | 3.5 | 0.035 | 0.1 | 3.4 | 3 |
| No anticoagulant | 11.6 | 3.6 | 0.03+ | <0.1 | 3.6 | <3 |
| With heparin | 10.9 | 3.6 | 0.035 | <0.1 | 3.6 | <3 |
| No anticoagulant | 10.5 | 15.2 | 0.14+ | 0.8 | 14.4 | 5 |
| With heparin | 9.8 | 13.8 | 0.14 | 0.9 | 12.9 | 7 |

Other workers have added lead salts to blood *in vitro* (7, 12), but have not given the actual data obtained. They stated only that the added lead was found in the cell fraction, without mentioning the quantities employed. Table 8 shows that when lead was added to normal rabbit blood (heparinized), most of the lead was found in the cell fraction, and that the ratio was approximately the same as that found in the blood of rabbits which were ingesting lead. When sufficient lead was added to the blood to be equivalent to a lead level of 0.35 mgm. per 100 grams, which was higher than that reached in any feeding experiment, at least 90% of the total lead was still found with the cells. However, when this added lead was increased so that the lead level reached 0.55 mgm. per 100 grams, the fraction of the total lead associated with the cells fell to 80%, which seems to indicate that the lead concentration of the cells reached a saturation value near that point. Some hemolysis occurred in all the samples reported in Table 8 (especially in the last one) so that the conditions were not exactly comparable to cases in which the lead had reached the blood through physiological absorption; this may explain why the lead extraction by the cells was not so complete as it is in the living animal.

TABLE 8

*Plasma-cell partition of lead added to rabbit blood in vitro*

| SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|
| | *grams* | *micrograms* | *mgm./100 grams* | *micrograms* | *micrograms* | |
| Normal blood: | | | | | | |
| No lead added | 10.4 | 1.3 | 0.01+ | 0.1 | 1.2 | 8 |
| Lead added | 10.7 | 9.4 | 0.09 | 0.9 | 8.5 | 10.5 |
| Lead added | 10.0 | 20.0 | 0.20 | 2.5 | 17.5 | 12.5 |
| Lead added | 11.5 | 40.4 | 0.35 | 3 | 37.4 | 7.5 |
| Lead added | 9.9 | 54 | 0.55 | 11 | 43 | 20 |

All workers who have studied the plasma-cell partition of lead in the blood have separated the two fractions obtained by centrifugation and have assumed that the lead found in the cell fraction was within the cells or on their surface. It seemed possible, although not probable, that the lead might be present in the blood in the form of a dispersed compound, either organic or inorganic, and that when the blood is centrifuged this compound, being heavier than the plasma, settles to the bottom part of the centrifuge tube with the cells. Washing the cells, the procedure which Blumberg and Scott (13) followed, does not exclude this possibility, for the cells are separated from their suspension in the washing medium by centrifuging the mixture, and the hypothetical lead compound could again follow them to the bottom.

In order to investigate this point, lead chloride solution was added to plasma obtained by centrifuging normal rabbit blood (heparinized), and after standing 15 minutes the mixture was centrifuged. It was found that the lead remained distributed throughout the plasma and did not settle out during this second centrifugation (table 9). This fact furnishes indirect evidence that the lead found in the cell fraction is actually associated with the cells.

Unexpectedly, it was found that when the cells were returned to plasma to which lead had been added they did not remove as much of the lead from the plasma as could be expected from the results of the addition of lead solution to whole blood. This is illustrated by the last two analyses in each experiment in Table 9, in that after the lower half of the plasma was mixed with the cells, it held as high a concentration of lead as did the cell fraction.

TABLE 9

*The effect of adding lead to the plasma of rabbit blood in the absence of blood cells*

| SAMPLE | WT. OF SAMPLE | TOTAL LEAD | LEAD |
|---|---|---|---|
| | *grams* | *micrograms* | *micrograms per gram* |
| I | | | |
| Cell fraction—no lead added | 6.7 | 1.5 | 0.2 |
| Top half of centrifuged plasma, lead added | 5.3 | 17.5 | 3.3 |
| Lower half of plasma, mixed with cells, then centrifuged—plasma portion | 3.7 | 6.9 | 1.9 |
| Cells from above mixture | 5.5 | 11.5 | 2.1 |
| II | | | |
| Cell fraction—no lead added | 4.8 | 1.0 | 0.2 |
| Top half of centrifuged plasma, lead added | 5.3 | 18.5 | 3.5 |
| Lower half of plasma, mixed with cells, then centrifuged—plasma portion | 5.0 | 13.0 | 2.6 |
| Cells from above mixture | 3.2 | 8.3 | 2.6 |

TABLE 10

*The combination of the cells of rabbit blood with lead under various conditions*

| SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|
| | *grams* | *micrograms* | *mgm /100 grams* | *micrograms* | *micrograms* | |
| Lead added directly to whole blood | 9.9 | 54 | 0.55 | 11 | 43 | 20 |
| Lead added to centrifuged remixed blood | 9.7 | 55.5 | 0.57 | 10.5 | 45 | 19 |
| Cells washed with lead-containing saline | | 20.9 | | 3.9* | | 19* |

* In saline.

The reduced ability of the cells to remove the lead may have been due either to some change induced in the cells by the experimental manipulations or to some type of combination of lead with the plasma. Table 10 indicates that the cells had not lost their power to take up lead. In these experiments normal rabbit blood (heparinized) was separated into plasma and cells by centrifugation, then the two fractions were mixed together thoroughly and after a few minutes the lead chloride solution was added to the recombined blood. After 15 minutes the blood was centrifuged again and the plasma and cell fractions were analyzed. In the last experiment in Table 10, cells from rabbit blood were washed with an equal volume of an isotonic saline solution containing lead chloride; here, also, most of the lead was removed by the cells.

The "combination" of the lead with the plasma, after admixture of the two, occurred rapidly; there was no significant difference between the lead partition found in experiments when cells were mixed with plasma immediately after the addition of a lead salt to the plasma and that obtained when about 10 minutes had been allowed to elapse before the introduction of the cells.

TABLE 11

*Plasma-cell partition of lead in rabbit blood after primary addition of lead to plasma alone*

| SAMPLE | WT. OF SAMPLE | TOTAL LEAD | TOTAL LEAD | LEAD IN PLASMA | LEAD IN CELL FRACTION | PER CENT OF TOTAL LEAD IN PLASMA |
|---|---|---|---|---|---|---|
| | *grams* | *micrograms* | *mgm./100 grams* | *micrograms* | *micrograms* | |
| Cells added to lead-containing plasma immediately, then separated after 15 minutes... | 9.1 | 22 | 0.24 | 10 | 12 | 45 |
| Cells added to lead-containing plasma after 10 minutes then separated after 15 minutes.... .............. | 8.2 | 21.3 | 0.26 | 10.5 | 10.8 | 49 |
| Cells added to lead-containing plasma after 10 minutes, then separated after 2 hours...................... | 9.0 | 22 | 0.24 | 10 | 12 | 45 |
| Cells added to lead-containing plasma immediately, then separated after 24 hours...... | 9.2 | 14.6 | 0.16 | 3.3 | 11.3 | 23 |
| Cells added to lead-containing plasma after 10 minutes, then separated after 24 hours | 9.8 | 13.9 | 0.14 | 3.7 | 10.2 | 27 |

It seemed probable that time would be a factor in any such reaction, and accordingly experiments were carried out in which the lead was added to the plasma, the cells stirred in, and the mixture then allowed to stand with agitation for two hours and for twenty-four hours. In the two-hour sample the concentration of lead in the cells was not significantly greater than that in the plasma, but in the twenty-four hour sample about three-fourths of the total lead was found in the cell fraction. Apparently, therefore, after sufficient time the cells will gradually remove lead from the plasma, even though the metal has previously entered into some type of combination with constituents of the plasma. Longer periods of equilibration were not tried because of the changes that occur in blood after it is shed. The results of these experiments are given in table 11.

In order to detect any possible absorption of lead by the leucocytes, a sample of heparinized blood from one of the lead-poisoned rabbits was separated into plasma, buffy layer, and red cell fractions. No significant difference was found between the lead concentration in the buffy layer and that in the red cell fraction, while the plasma, as usual, contained only a slight amount of lead.

Discussion. The results reported in this paper are in substantial agreement with those obtained with a dithizone method by Willoughby and Wilkins (15) and spectrographically by Blumberg and Scott (13), and by Kehoe, Cholak, and Story (16). They differ from the figures given by Tompsett and Anderson (17), Teisinger (12), Schmitt and Taeger (14), and Aub, Fairhall, Minot, and Reznikoff (6). Tompsett and Anderson, using potassium oxalate as an anticoagulant, found about one-third of the total lead in the plasma in human blood from normal individuals and also from those with lead poisoning. When working with normal rabbits and animals receiving lead orally, they found from one-third to more than one-half of the total blood lead in the serum. It is probable that the disagreement between their results and ours is due to differences in the analytical methods used. In addition, it should be pointed out that the conclusions stated here are based upon a large number of closely agreeing analyses, obtained by a method of proved specificity and accuracy.

The combination of lead salts with blood plasma so as to interfere with their usual behavior has been noticed by others. According to Minot (24), when red cells are exposed to lead which has been previously mixed with serum the characteristic effects of the lead on fragility and stickiness of the cells are no longer produced. Aub and Reznikoff (25), and Orskov (26) believe that the substance in serum that reacts with lead is inorganic phosphate. There seems to be little or no actual evidence to substantiate this belief.

The work of Kety (27) may be reviewed in the light of the evidence presented here that little or no lead is present in the blood plasma or serum. Kety's conclusions were based upon ionic relationships in plasma and serum; he states that the normal blood citrate (in human plasma) is capable of keeping in solution an amount of lead 20 times as great as that which the solubility of the insoluble lead compounds of the body would otherwise permit. In view of the analytical results reported here these conclusions may lose some of their significance.

## SUMMARY

*In an investigation of the plasma-cell partition of blood lead the following* conclusions were reached:

(1) At least 90% of the total blood lead of rabbits is found in the cell fraction after separation of the latter by centrifugation; this applies both to normal animals and to those exposed to large quantities of lead. The same is true in the case of human blood.

(2) It is probably correct to assume that the lead found in the cell fraction after centrifugation of rabbit blood is there by virtue of an actual association with the cells, and not as the result of the formation of a lead compound which settled out with the cells during the centrifugation. If some lead compound of this type is present in blood it is formed only in the presence of the cells, not the plasma alone.

(3) There is no significant difference between the plasma-cell lead partition in arterial blood and that in venous blood.

(4) The use of heparin, potassium oxalate, or sodium citrate does not cause a significant change in the plasma-cell lead partition.

(5) The plasma-cell and the serum-clot partitions of lead in rabbit blood do not differ significantly.

(6) When lead chloride solution is added to the whole blood (heparinized) of normal rabbits most of the lead is taken up by the cells; over 90% of the total lead is found in the cell fraction with lead levels as high as 0.35 mgm. of lead per 100 grams of blood, while at 0.55 mgm. per 100 grams at least 80% of the lead is associated with the cells.

(7) When lead chloride solution is added to the heparinized plasma of the blood of normal rabbits the reactivity of the lead is interfered with in some way by the plasma, so that when the cells are returned to the plasma, their removal of the lead is retarded; 24 hours are needed for the removal of as much as 75% of the total lead.

(8) This reduced ability of the cells to remove lead which has been added to blood plasma is not due to changes suffered by the cells during centrifugation, but is caused by some type of reaction of the lead with the plasma.

(9) These facts indicate that there is a rapid reaction between lead and some constituent of the cells, taking precedence over the reaction that occurs in plasma in the absence of cells. Presumably, the reaction with the cells results in the formation of a more insoluble—or less reactive—compound than that which is formed with the plasma.

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# A STUDY OF THE SIMILARITIES OF SEVERAL REPRESENTATIVE TYPES OF BISMUTH PREPARATIONS USED IN THE THERAPY OF EXPERIMENTAL SYPHILIS

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Since the time of the introduction of bismuth into the therapy of syphilis many different preparations have been employed with various advocates stressing the superiority of one or another of the many types. In general, considerable emphasis is, and has been, placed upon the relative safety, efficiency, and freedom from annoyance to the patient. In the experimental field, efficiency is usually expressed in terms of the therapeutic index. The mode of administration is generally quite important in respect to toxicity, as is proven by the fact that when bismuth preparations are administered intravenously all are extremely dangerous, while on intramuscular injection tolerance is, as a rule, very much higher. The well-known differences in rates of mobilization of different preparations from intramuscular deposits have often led to misconceptions regarding the significance of this factor. The rates of absorption from intramuscular deposits, the rates of elimination and intermediary dissociation of bismuth, in some form or other, from the original complex, are obviously all basic factors bearing on the question of toxicity. It was therefore the problem of the writers to analyze some of the various factors bearing on the questions of toxicity and therapeutic efficiency. Especial attention has been paid to similarities as well as differences between certain representative members of the various types of bismuth preparations.

The five bismuth preparations discussed in a preliminary report (1) from this laboratory were used in this study and are taken to be representative of the various types.[1] They are thiobismol and bismuth sodium citrate, both soluble in water and very rapidly absorbed from intramuscular deposits, bismuth sodium tartrate, also water-soluble, iodobismitol, rather more slowly absorbed, and bismuth ethyl camphorate, an oil-soluble preparation, rather slowly absorbed. Of the five, all but thiobismol are precipitated in tissues following intramuscular injection and precipitated in serum *in vitro*.

As stated above, the usual basis for comparing two or more agents in specific chemotherapy is to determine their therapeutic indices (*T.I.*), which are the ratios of maximal tolerated (*M.T.D.*) to the minimal curative doses (*M.C.D.*). Owing to the well-known fact that the rate of absorption from intramuscular deposits is more or less specific and variable for each of the various bismuth preparations, it seemed highly desirable to avoid this basic difference by employment of the intravenous route of administration of these drugs in one phase of this work. Data obtained from such experiments are then available for comparison with corresponding data obtained by the intramuscular route of administration.

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[1] We wish to thank Parke, Davis & Company for the thiobismol, G. D. Searle & Company for the bismuth sodium tartrate, and The Upjohn Company for the bismuth sodium citrate and the bismuth ethyl camphorate.

TOXICITY STUDIES. For the determination of the M.T.D. of each preparation healthy rabbits of about 2 kilograms body weight were employed. They were kept under uniform housing conditions and fed on a diet of alfalfa and oats. The intravenous M.T.D. was determined by single injections into the marginal ear veins at the rate of 1 mgm. of bismuth, as metal, per minute. When this rate of injection was strictly adhered to there were no immediate deaths ascribable to flocculation or embolism but instead, with a fatal dose, death occurred in from three to seventeen days. Tissues obtained at autopsies revealed the typical renal damage characteristic of subacute bismuth poisoning. For the determination of the M.T.D. by intramuscular administration the preparations were injected in relatively small volume into the thigh muscles. The M.T.D. were determined as those doses at which 66% of the animals survived for twenty days. From three to six animals were employed at each dose.

Bismuth sodium tartrate, bismuth ethyl camphorate, and iodobismitol, administered intravenously, were found to be tolerated at essentially the same dosages whereas bismuth sodium citrate and thiobismol apparently were slightly more toxic (Table 1). This slight difference between the two groups may possibly be accounted for by the fact that there are some differences in solubility in serum, the less soluble preparations being removed by the reticulo-endothelial tissues. The more soluble forms would be less subject to the formation of minute particles and hence would remain in the blood stream for a longer time to be concentrated in the organ of excretion, namely, the kidney. A recent report by Sollmann and Seifter (2) substantiates this interpretation.

In contrast to the marked similarity of toxicity by the intravenous route of administration, these same preparations differ very greatly with respect to intramuscular toxicity (table 1). Thiobismol appeared to be essentially as toxic when administered intramuscularly as it was when given intravenously. At the other extreme, the tartrate was approximately fifteen times more toxic intravenously than when given intramuscularly.

THERAPEUTIC STUDIES. Healthy male rabbits weighing approximately 2 kilograms were inoculated with the Nichols' strain of *Treponema pallidum*. This strain has been maintained in this laboratory since 1923 by passage through rabbits at intervals of six to eight weeks. The inoculations were made into the testes; the inoculum was prepared in the usual manner from fresh chancre material from an infected rabbit and was of such concentration that 0.5 cc. of this suspension caused lesions to appear in from five to six weeks. Serological tests were made at the Wisconsin Psychiatric Institute, under the direction of Dr. W. F. Lorenz; the test used was especially adapted to serological reactions of the rabbit.

Only rabbits with typical lesions were chosen on the sixth to eighth week after inoculation. In the determination of the M.C.D. of each compound two procedures were employed, namely, the single and multiple dose system. This was done for purposes of comparison. The animals were treated by injecting the bismuth preparation either intramuscularly or into the marginal ear vein at the rate of 1 mgm. of bismuth, as metal, per minute. The single M.C.D. was taken as that dosage which when given in one injection cured 66% of the animals, while the multiple M.C.D. was taken to be that dose which when given three times at weekly intervals cured the same percentage of the animals. From one to five animals were used at each dose. As a criterion of cure, popliteal lymph nodes from each treated animal were transferred, three weeks after the last treatment, into the testis of each of two recipient animals. Only rabbits with negative serological reactions were employed as recipients, which were then observed for from eight to twelve weeks for the development of lesions or positive serological reactions.

TABLE 1

*Summary of maximal tolerated (M.T.D.) and minimal curative (M.C.D.) doses and therapeutic indices (T.I.)*

Dosages are expressed as milligrams of bismuth metal per kilogram body weight

| PROCEDURE | BISMUTH SODIUM TARTRATE | BISMUTH ETHYL CAMPHORATE | IODO-BISMITOL | BISMUTH SODIUM CITRATE | THIOBISMOL |
|---|---|---|---|---|---|
| *M.T.D.* | | | | | |
| Single injection | | | | | |
| Intravenous | 4.0 | 5.0 | 5.0 | 3.0 | 1.5 |
| Intramuscular | 59.0 | 40.0 | 20.0 | 10.0 | 2.0 |
| *M.C.D.* | | | | | |
| Intravenous | | | | | |
| Single | 3.0 | 3.0 | 3.0 | 3.0 | — |
| Multiple (×3) | 1.5 | 1.5 | 1.5 | 1.5 | 1.0 |
| Total multiple | 4.5 | 4.5 | 4.5 | 4.5 | 3.0 |
| Intramuscular | | | | | |
| Single | 3.0 | 3.0 | 3.0 | 3.0 | — |
| Multiple (×3) | 1.0 | 2.0 | 2.0 | 1.0 | 1.5 |
| Total multiple | 3.0 | 6.0 | 6.0 | 3.0 | 4.5 |
| *T.I.* | | | | | |
| Intravenous | | | | | |
| Single | 1.3 | 1.7 | 1.7 | 1.0 | — |
| Multiple | 2.7 | 3.3 | 3.3 | 2.0 | 1.5 |
| Intramuscular | | | | | |
| Single | 19.6 | 13.0 | 6.5 | 3.3 | |
| Multiple | 59.0 | 20.0 | 10.0 | 10.0 | 1.3 |

As may be seen from table 1, the M.C.D. of each drug, with the exception of thiobismol, was essentially the same whether given intravenously or intramuscularly, or as single or multiple doses. The therapeutic index (M.T.D./M.C.D.) in all of the intravenously treated animals and for all the drugs, except thiobismol, was slightly over one. On the other hand, there exists a marked difference in M.T.D., and therefore in the therapeutic indices, when the drugs are given intramuscularly. Thiobismol, apparently owing to its rapid absorption, is not well enough tolerated to cure in a single dose but does so in three slightly smaller doses given at weekly intervals.

It is to be noted that all curative doses are of the same order of magnitude regardless of the route of administration. Again, it is to be seen that, intravenously administered, the M.T.D. are all of the same order of magnitude. There are slight differences in readiness of dissociation, as indicated by readiness of production of flocculation when put directly into serum, which fact may well account for the minor inequalities in toxicity. The one clear-cut and distinctive differential feature of the various preparations is in the M.T.D. when they are administered intramuscularly. The therapeutic index, and hence the therapeutic margin of safety, is greatest in those preparations which, when given intramuscularly, are best tolerated. Thus, the intramuscular injection, as a mode of administration, offers the greatest safety for most of the preparations. One of them, thiobismol, as mentioned above, appears to be as toxic intramuscularly as intravenously, hence yields the same therapeutic index by either route of administration. It would follow, also, that both curative and toxic reactions, in the present series, are dependent upon or proportional to the content of elemental bismuth. This inference is supported in part by the observations of Eagle (3) and of Kolmer and his associates (4) in their study of the therapeutic action of various preparations of bismuth on spirochetal suspensions *in vitro*.

Other investigators have presented a very considerable amount of data on the M.C.D. of various bismuth preparations given intramuscularly in the therapy of experimental syphilis (5, 6, 7, 8). An analysis of these data reveals that when the dosages are reduced to terms of metallic bismuth the curative dosages mostly fall into an order of magnitude similar to the figures presented above. Kolle (7) and Klauder (8) have reported data on intravenous therapy using other preparations which also fall within the ranges of our own figures. Hence a correlation of such data would appear to support indirectly the conclusion presented herein.

The basic similarity of all of these bismuth preparations is indicated furthermore in a recent report from this laboratory (9), where it was found that of the three preparations studied, all were therapeutically additive when given with mapharsen. Thus, one-fourth, one-half or three-fourths of 1 milligram of elemental bismuth, in the compounds reported upon, when co-acting with three-fourths, one-half or one-fourth, respectively, of 1 milligram of mapharsen, each per kilogram, are curative combinations for syphilis in the rabbit.

It may be of practical interest to mention, incidentally, that mapharsen has the same M.C.D., *expressed as compound*, in rabbit syphilis as have the various bismuth compounds when the dosages of these are expressed in terms of elemental bismuth. It is evident, then, that therapeutically in rabbit syphilis, 1 milligram of elemental bismuth, in any of the forms employed in this study, happens to be equivalent to 1 milligram of mapharsen.

## SUMMARY

(1) There exists a striking qualitative and quantitative similarity of toxicities or limits of tolerance as well as of antisyphilitic potencies among the various bis-

muth preparations when they are administered intravenously. When they are administered intramuscularly, the antisyphilitic action does not differ significantly from that seen on intravenous injections, even though the tolerance by the host for most of the preparations is markedly increased.

(2) The similarity of the M.T.D. and the T. I. of the compounds when administered intravenously, in contrast to their marked differences when given intramuscularly, *indicates that the difference in rate of absorption from intramuscular deposits is the chief and most important factor accounting for any variation in behavior which does appear.*

(3) From the results obtained in this study and from a survey of reports by others, it is evident that all of the bismuth preparations, by and large, manifest *their therapeutic activity as well as toxicity in direct proportion to their elemental bismuth content.*

(4) The similarity of the M.C.D. of any preparation of bismuth by either route of administration and the similarity in the toxicity of any of the bismuth preparations when given intravenously suggest that basically all preparations of bismuth *ultimately act in a common form rather than the form in which they are injected.*

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# CHANGES IN THE BLOOD PICTURE OF THE DOG FOLLOWING SUBCUTANEOUS INJECTIONS OF SODIUM SELENITE[1]

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Acute selenium poisoning in range livestock has often been called "Blind Staggers" and is not uncommon among range animals in seleniferous areas, especially when they are being moved from one range to another. Usually grasses are among the first range plants to dry up, leaving the *Astragali* and other highly seleniferous plants in increased proportion of the succulent plants on the range. Even though many of these plants have a strong characteristic odor, and are not eaten under normal range conditions, animals will often eat them when they are hungry. This is especially true when the herd is moving (spot grazing) and there is not enough time to discriminate between the toxic and non-toxic plants. The symptoms accompanying "Blind Staggers" have been adequately described (1, 2, 3) but they may be briefly summarized as (in the last stages): varying degrees of paralysis, abdominal pain, grating of teeth, salivation and grunting. Death usually results from failure of respiration.

A method of treating such animals would be of considerable value. However, knowledge of the physiological effects of selenium salts is a prerequisite to the development of such a procedure.

Probably the first report of the toxicity of selenium compounds for the laboratory animal was that of Gmelin (4). He observed that selenium was very toxic and that shortly after its administration the animal gave off a garlic-like odor in its breath. In a review of the early work on selenium toxicity, Jones (5) states that as this garlic-like odor appeared, animals became restless, mouthing their tongues and vomiting. Somnolence appeared, gave way to unconsciousness and ultimate death. These gross symptoms have been confirmed with several species of animals including the rat, which does not vomit, but does undergo spasmodic contractions of the flanks which distress it greatly (Franke and Moxon (6)).

Franke and Moxon (6) also reported that intraperitoneal injection of sodium selenite at a level of 3.25–3.5 mgm./kgm. of body weight was sufficient to kill 75% of young rats. Sodium selenate was not as toxic as the selenite since about 5.25–5.75 mgm./kgm. were required to give the same per cent of deaths. Smith, Stohlman and Lillie (7) reported that among the common laboratory animals the rat was the most resistant to selenium poisoning while the cat was least resistant. They reported no difference in the degree of toxicity of selenite or selenate when injected intravenously, 1.5–3.0 mgm./kgm. being the minimum lethal dose for rabbits, cats and rats. They likewise reported that the method of administration, feeding or injection, made little difference on the degree of toxicity. Work in this laboratory would indicate that animals which receive selenium orally or by intraperitoneal injections are able to withstand higher dosages than those which receive subcutaneous injections. This applies to both rats and dogs.

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As early as 1935 Painter (unpublished data) observed that when rats were injected with lethal doses of selenium, their hemoglobin levels sometimes increased from 15 to 25 grams per 100 cc. of blood. This was confirmed in dogs by Moxon in the same year (unpublished).

Such a profound change in the hemoglobin concentration soon prompted the question, "What happens to the other blood constituents?" Since rats are too small to supply adequate blood samples for detailed analysis dogs have been used as experimental animals. In this report we present the results of investigations on the effect of injected selenium salts on the blood picture of dogs together with data which indicate the minimum fatal dose of sodium selenite for the dog.

EXPERIMENTAL. Actions of the dog due to the severe griping and pain resulting from selenium administration make it very difficult to obtain blood samples at regular intervals of time and it is especially difficult to make blood pressure measurements. Therefore these studies were made on dogs which were under barbital depression. The drug was administered orally in capsules at a level of 150 mgm./kgm. of body weight. In addition ether anesthesia was employed for just the time necessary to cannulize the femoral artery. Blood samples were usually withdrawn from the jugular vein with a hypodermic needle, although the fall in blood pressure just preceding death made it necessary in some cases to take the last samples directly from the heart. As a general policy, blood samples were taken immediately before the administration of barbital and at 30 minute intervals thereafter. The selenium salt was injected subcutaneously above the shoulder 60 or 90 minutes later depending upon the time necessary for the full effect of the barbital. Thus, changes in the blood picture before selenium was injected indicate the effect of barbital and brief ether anesthesia. As a further check on the effects of barbital and ether, control animals (receiving no selenium) were also studied.

*Effect on the blood picture.* Analyses of the blood included the following determinations: hemoglobin, determined as oxyhemoglobin by the method of Evelyn and Malloy (8); inorganic phosphorus by the method of Fiske and Subbarow (9), adapted for use on the photoelectric colorimeter; ascorbic acid, by the method of Mindlin and Butler (10); calcium, by the method of Clark and Collip (11); glutathione, by the method of Benedict and Gottschall (12); blood sugar, by the method of Folin and Malmros (13); non-protein nitrogen, by the method of Folin and Wu (14); and selenium, by the method of Klein (15). Not all of these determinations were made on each sample from each dog because of the limit to the size of the samples when they are taken at 30 minute intervals. Normal or control estimations were made on blood samples taken from most of the dogs before they had received any selenium.

All dogs were in good health and had been maintained on our stock dog ration for a few weeks before they were used for these studies. This ration has the following composition: cooked corn meal 72%; tankage 18%; lard 5%; salts 3%; cod liver oil 1%; yeast 1%.

Table 1 summarizes the effect on the blood picture of dog no. 1. This dog was under barbital depression throughout the experiment and ether anesthesia while the cannula was being inserted. Sodium selenite was injected at a level of 2.5 mgm. Se per kilogram. No blood sample was taken from this dog before the selenium injection.

After 30 minutes the dog exhibited nausea and vomiting. Ten minutes later a copious discharge of serous fluid from the respiratory tract was observed. At 55 minutes the blood pressure fell very sharply, just before the animal died. On autopsy there was observed a beading of the smaller blood vessels (fig. 1) throughout the mesenteric area. Also there was some fluid in the thoracic cavity.

TABLE 1

*Changes in the blood of dog no. 1 which received sodium selenite equivalent to 2.5 mgm. selenium/kgm. body weight; mature dog, weight 25.2 kilograms*

| DETERMINATION | SAMPLE | MINUTES AFTER INJECTION | | |
|---|---|---|---|---|
| | | 0 | 30 | 55 |
| Hemoglobin (grams/%) | Whole blood | 17.4 | 19.2 | 28.2 |
| Hematocrit (% cells) | Whole blood | 45 | 50 | 68 |
| Ascorbic acid (mgm %) | Plasma | .503 | 1.120 | 443 |
| Inorganic P (mgm %) | Whole blood | 1.80 | 1.36 | 1.18 |
| Blood sugar (mgm. %) | Whole blood | 87.3 | 83.0 | 56.7 |
| Non-protein nitrogen (mgm. %) | Whole blood | 94 | 39.2 | 26.4 |
| Selenium (p.p.m.) | Whole blood | 0.0 | 5.2 | 6.0 |
| Blood pressure (mm. of Hg) | | 140 | 113 | 65–0 |

Survival time after selenium injection, 55 minutes.

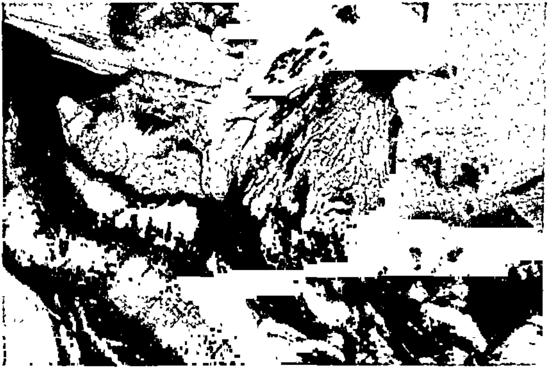

FIG. 1. PHOTOGRAPH OF THE MESENTERIC AREA OF A DOG WHICH HAS RECEIVED A FATAL DOSE OF SELENIUM (2.5 MGM./KGM.) AS $Na_2SeO_3$

Note the engorgement of the larger blood vessels and the beading in the smaller vessels

Blood analyses showed a progressive rise in hemoglobin values from 17.4 to 28.2 grams per 100 cc. of blood, an increase of 62%. Hematocrit values showed a similar trend, rising from 45 to 68%. Ascorbic acid values rose sharply and then fell. Initial inorganic phosphorus values were low, but they fell even lower after selenium administration. Blood sugar dropped from 87.3 to 56.7 mgm. per 100 cc. and non-protein nitrogen dropped from 94 to 26.4 mgm. per 100 cc. The selenium concentration of the blood reached a level of 5.2 parts per million at the time nausea and vomiting were observed and rose to 6.0 just before the animal died. It has been found that this result is typical of the dog which has received slightly more than a minimum fatal dose of selenium administered subcutaneously as sodium selenite.

To determine the effect of barbital and ether on the blood picture of the dog, a control animal (dog no. 5) was subjected to the same treatment as dog no. 1 except that it received no selenium. Table 2 summarizes data from this dog. Since the variations observed for this animal do not follow the trends for the selenized animal it is possible to differentiate the two. The results on this animal (dog no. 5) were typical of other animals used as controls. Vitamin C and inorganic phosphorus data for two other control dogs under barbital depression are given in table 6. The hemoglobin concentration, hematocrit, and blood pressure remained unchanged while inorganic phosphorus exhibited a slight rise. The greatest effect was shown in the marked rise in non-protein nitrogen. These effects are directly opposed to those seen in a selenized dog.

Some dogs showed a resistance to selenium poisoning, that is, the onset of death was delayed and the concentration of the formed elements of the blood was not as marked. The effect of selenium (2.25 mgm. per kilogram) on the blood picture of such a resistant animal (dog no. 4) is shown in table 3. In this case the hemoglobin increased only 13.3% (from 19.5 to 22.1 grams per 100 cc.) as compared with an increase of 62% for dog 1. Hematocrit values increased from 51.0 to 55%. Most significant was the marked rise in ascorbic acid in the plasma, an increase from 0.72 to 1.575 mgm. per 100 cc. The inorganic phosphorus in the blood of this dog dropped significantly before the selenium was injected and remained very low throughout the experiment. The greater survival time after Se injection shown by this dog in contrast to the very short survival of dog no. 1 was notable. Dog no. 3 (table 4) received less Se (2.0 mgm./kgm.) but survived only 120 minutes. The ascorbic acid level in the blood of dog no. 3 was much lower at the time of selenium injection and even though it did double in concentration it did not reach the initial level of dog no. 4.

One dog (no. 6) failed to exhibit the typical rise in hemoglobin and hematocrit but showed a very striking rise in ascorbic acid (table 5). In this dog the blood sugar, instead of increasing, decreased from 124 to 101 mgm.% in the plasma. The level of inorganic phosphorus and non-protein nitrogen decreased as usual and the blood pressure curve was typical.

Since two of the resistant dogs showed high levels of ascorbic acid in their blood, we next tried injecting ascorbic acid to raise its concentration in the blood in an effort to increase the resistance of the dogs. The ascorbic acid was dissolved in M/15 phosphate buffer at pH 7.2. The resulting solution contained 20

TABLE 2

*Changes in the blood of dog no. 5 which received no selenium, serving as a control to establish the effects of barbital and ether; mature dog, weight 11.8 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0* | 30 | 60 | 90 | 135† | 165 | 195 | 255 | 315 |
| Hemoglobin (grams/%) | Whole blood | 15.8 | 14.7 | 14.2 | 13.6 | 14.0 | 14.2 | 15.6 | 16.1 | 15.0 |
| Hematocrit (% cells) | Whole blood | 38.5 | 17.5 | 37.0 | 35.5 | 34.5 | 34.5 | 38.0 | 40.0 | 33.0 |
| Ascorbic acid (mgm. %) | Plasma | .540 | .540 | .605 | .508 | .282 | | .572 | .734 | .410 |
| Inorganic P (mgm. %) | Whole blood | 3.23 | 3.43 | 3.23 | 3.46 | 3.92 | 3.85 | 3.96 | 4.78 | 3.98 |
| Blood sugar (mgm. %) | Whole blood | 124 | 128 | 178 | 149 | 192 | 200 | 200 | 213 | 167 |
| Non-protein nitrogen (mgm. %) | Whole blood | 109 | 132 | 118 | 133 | 125 | 140 | 125 | 198 | 242 |
| *Blood pressure (mm. of Hg)* | | | . | | | | 110 | 113 | 113 | 115 |

* Barbital administration equivalent 150 mgm./kgm.

† Ether anesthesia and insertion of cannula.

TABLE 3

*Changes in the blood of dog no. 4 which received sodium selenite equivalent to 2.25 mgm. selenium/kilogram; mature dog, weight 24.1 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 30 | 60* | 120† | 150 | 180 | 210 | 270 | 330 |
| Hemoglobin (grams/%) | Whole blood | 18.2 | 18.2 | 18.9 | 19.5 | 18.7 | 19.0 | 19.7 | 20.0 | 22.1 |
| Hematocrit (% cells) | Whole blood | 46.5 | 46.5 | 48.5 | 51.0 | 47.4 | 48.8 | 51.0 | 52.5 | 55.5 |
| Ascorbic acid (mgm. %) | Plasma | .151 | .227 | .075 | .072 | .270 | .518 | .625 | 1.35 | 1.575 |
| Inorganic P (mgm. %) | Whole blood | 2.67 | 2.16 | | 1.52 | 0.96 | 0.87 | 0.57 | 1.05 | 0.98 |
| Blood sugar (mgm.%) | Whole blood | 97 | 111 | 100 | 141 | 116 | 93.5 | 77.6 | 78.0 | 58.0 |
| Non-protein nitrogen (mgm. %) | Whole blood | 88.1 | 84.0 | 78.0 | 73.5 | 72.0 | 66.5 | 76.1 | | |
| Selenium (p.p.m.) | Whole blood | | | | 0 | 1.3 | 1.7 | 1.8 | 1.7 | 1.6 |
| Blood pressure (mm. Hg) | | | | 120 | 120 | 115 | 116 | 117 | 119 | 92–0 |

* Ether anesthesia and cannulization.

† Received $Na_2SeO_3$ equivalent to 2.25 mgm. selenium/kgm.
Survival time after Se injection 220 minutes.

mgm. of ascorbic acid per cc. and served a dual purpose by furnishing phosphate as well as ascorbic acid.

The results with the first dog (11B) to receive the ascorbic acid are shown in table 7. Ninety minutes after barbital administration the dog received an injection of ascorbic acid. Thirty minutes later sodium selenite was injected. Noteworthy is the fact that the hemoglobin concentration did not go above 16.8 grams per 100 cc. The level of ascorbic acid was at its maximum from 30 to 60 minutes after it was injected. Then the concentration decreased until just before the animal died when it rose sharply. This dog lived 255 minutes after the administration of the selenite while dog no. 3 (table 4) which received the same amount of selenium per kilogram of body weight but no injection of ascorbic acid lived only 120 minutes.

TABLE 4

*Changes in the blood of dog no. 3 which received sodium selenite equivalent to 2.0 mgm. selenium/kilogram; mature dog, weight 22.3 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES AFTER Se INJECTION | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 | 105 | 120 |
| Hemoglobin (grams/%) | Whole blood | 17.6 | 18.2 | 19.3 | 22.1 | 23.0 | 25.0 |
| Hematocrit (% cells) . . | Whole blood | 39.0 | 45.5 | 48.0 | 54.5 | 59.0 | 62.0 |
| Ascorbic acid (mgm. %) | Plasma | 0.237 | .388 | .432 | .796 | .612 | .464 |
| Blood sugar (mgm. %). | Whole blood | 147 | 108 | 96 | 89.6 | 74.5 | 79.5 |

Survival time after selenium injection 120 minutes.

TABLE 5

*Changes in the blood of dog no. 6 which received sodium selenite equivalent to 2.25 mgm. selenium/kilogram; mature dog, weight 17.80 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES AFTER Se INJECTION | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 | 120 | 150 | 160 |
| Hemoglobin (grams %)............... | Whole blood | 13.2 | 14.2 | 15.0 | 15.3 | 16.2 | 16.5 | 15.9 |
| Hematocrit (% cells). | Whole blood | 35.0 | 35.5 | 38.0 | 38.0 | 41.5 | 42.5 | 36.5 |
| Ascorbic acid (mgm. %)............... | Plasma | .614 | .722 | .885 | 1.240 | 1.135 | 1.500 | 1.675 |
| Inorganic P (mgm. %) | Whole blood | 3.87 | 3.36 | 2.77 | 2.87 | 2.68 | 2.90 | 3.18 |
| Blood sugar (mgm. %) | Whole blood | 124 | 122 | 118 | 106 | 109 | 101 | 103 |
| Non-protein nitrogen (mgm. %)......... | Whole blood | 61.2 | 61.2 | 79.0 | 62.5 | 33.0 | 37.5 | 40.0 |
| Selenium p.p.m...... | Whole blood | 0 | 3.8 | 2.3 | 3.0 | 2.0 | 3.1 | |
| Blood pressure, mm. Hg............... | | 113 | 119 | 111 | 112 | 102 | 90 | 65 |

Survival time after selenium injection 160 minutes.

The next dog, no. 12 B (table 8), was given ascorbic acid 30 minutes after the injection of sodium selenite. Under the influence of selenite the hemoglobin rose from 11.1 to 15.1 grams %, but when ascorbic acid was injected it decreased again to 14.1 grams% and remained at about that level for at least 7 hours. The ascorbic acid may have delayed the onset of death since this dog did not die until 2 days later.

TABLE 6

*Changes in the blood of dog no. 13 which received sodium selenite equivalent to 2.5 mgm. selenium/kilogram*

Survival time after selenium injection 120 minutes
Young dog, weight 10.12 kilograms

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 30 | 60* | 90 | 120 | 150† | 180† |
| Hemoglobin (gms./%)… | Whole blood | 17.0 | 16.1 | 14.6 | 15.5 | 17.2 | | 24.8 |
| Inorganic P (mgm. %)… | Plasma | 3.1 | 5.3 | 6.2 | 4.4 | 3.7 | 4.7 | 5.8 |
| Ascorbic acid (mgm. %)… | Plasma | .201 | .243 | .207 | .266 | | .504 | .645 |
| Glutathione (mgm. %)… | Whole blood | 51.4 | 51.4 | 49 4 | 24.6 | 30.6 | 63.0 | 83.2 |

*Changes in the blood of dog no. 11 which received no selenium (control)*

Young dog, weight 7.3 kilograms

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 | 150 |
| Ascorbic acid (mgm. %) .. | Plasma | .414 | .298 | .402 | .388 | .394 |
| Inorganic phosphorus . | Plasma | 4 47 | 4.05 | 4.82 | 4.60 | 3.90 |

*Changes in the blood of dog no. 12 which received no selenium (control)*

Mature dog, weight 16.8 kilograms

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 | 150 |
| Ascorbic acid (mgm. %) .. | Plasma | .392 | .410 | .594 | .630 | .622 |
| Inorganic phosphorus.... | Plasma | 4.23 | 4.08 | 5.02 | 4.92 | 4.73 |

* Selenium injected, dog No. 13.

† The last 2 samples from dog No 13 were drawn by heart puncture.

Dog no. 14 (table 9) was given more than a minimum fatal dose of sodium selenite (2.5 mgm. Se/kgm.). It has been our experience (table 1) that dogs which receive this high dosage die within an hour following the injection. With this dog the administration of ascorbic acid was begun 30 minutes before selenium injection and repeated every 30 minutes in an effort to build up and maintain a high level in the blood. The dog died 2½ hours after the selenium injection, showing that the ascorbic acid in the phosphate buffer was not able completely to offset the toxic effects of selenium at the level used. However, it did appear to prolong the life of the dog, since other dogs which have been injected with this high level of sodium selenite have died soon after symptoms of suffocation were observed, while this dog showed symptoms of suffocation for 90 minutes before death. More data are necessary to confirm this observation, but the extended period in which the animal was apparently suffocating before death was very striking.

TABLE 7

*Changes in the blood of dog no. 11B which received an injection of ascorbic acid in $\frac{M}{15}$ phosphate buffer followed after 30 min. by sodium selenite equivalent to 2.0 mgm. selenium/kilogram; mature dog, weight 7.26 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 50 | 90* | 120† | 150 | 180 | 210 | 240 | 330 |
| Inorganic P (mgm. %)... | Plasma | 4.95 | 4.80 | 4.14 | 4.70 | 3.89 | 1.90 | 1.25 | 1.25 | 1.20 |
| Hemoglobin (grams %)... | Whole blood | 14.2 | 13.2 | 11.5 | 12.3 | 15.0 | 14.8 | 14.7 | 14.0 | 16.8 |
| Ascorbic acid (mgm. %).... | Plasma | .298 | .445 | .570 | .916 | .914 | .708 | .648 | .353 | .927 |

* Ascorbic acid equivalent 5 mgm./kgm. in $\frac{M}{15}$ phosphate buffer at pH = 7.2.

† $Na_2SeO_3$ injected.

Survival time after selenium injection 255 minutes.

TABLE 8

*Changes in the blood of dog no. 12B which received sodium selenite equivalent to 2 mgm. selenium/kgm. followed after 30 min. by ascorbic acid in $\frac{M}{15}$ phosphate buffer; mature dog, weight 16.83 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 50 | 90* | 120† | 150 | 180 | 210 | 270 | 330 |
| Inorganic P (mgm. %)... | Plasma | 3.42 | 3.06 | 2.91 | 3.40 | 3.02 | 2.65 | 2.39 | 2.11 | 2.90 |
| Hemoglobin (grams %)... | Whole blood | 13.9 | 12.9 | 11.1 | 15.1 | 14.1 | 14.2 | 13.9 | 12.1 | 13.9 |
| Ascorbic acid (mgm. %)... | Plasma | .344 | .456 | .453 | .494 | .864 | .720 | .667 | .488 | .805 |

* $Na_2SeO_3$ injected.

† Ascorbic acid equivalent to 5 mgm./kgm.

The dog died two days later.

*Minimum fatal dose.* Results from 23 dogs indicate that subcutaneous injection of sodium selenite at a level of 2.0 mgm. of selenium per kgm. body weight was fatal to over 85% of the animals. Levels of 1.5 mgm./kgm. killed only occasionally. If, however, 1.5 mgm./kgm. were given followed by 0.5 mgm./kgm. after 24 hours, the animals invariably died within the next 48 hours. Thus, the minimum fatal dose of selenium for the dog lies between 1.5 and 2.0 mgm. per kilogram of body weight when injected subcutaneously. See table 10.

TABLE 9

*Changes in the blood of dog no. 14 which received sodium selenite equivalent to 2.5 mgm. selenium/kgm. with injections of ascorbic acid in* $\frac{M}{15}$ *buffer at 30 min. intervals until death; young dog, weight 12.11 kilograms*

| DETERMINATION | SAMPLE | TIME IN MINUTES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 30 | 60* | 90† | 120 | 150 | 180 | 210 | 240‡ |
| Inorganic P (mgm. %).. | Plasma | 5 75 | 6.00 | 6.23 | 6.85 | 6.18 | 5.41 | 4.29 | | |
| Hemoglobin (grams %)... | Whole blood | 16.3 | 15.0 | 13 0 | 12.5 | 15 8 | 19.0 | 20.2 | 20.7 | 25.9 |
| Ascorbic acid (mgm. %).... | Plasma | 382 | .805 | .865 | .920 | .821 | .683 | .642 | 1.270 | .292 |

* Ascorbic acid equivalent to 5 mgm./kgm. repeated every 30 minutes.

† $Na_2SeO_3$ injected.

‡ The last sample was taken by heart puncture.

Survival time after selenium injection 150 minutes.

TABLE 10

*Summary of data on minimum fatal dose of selenium (sodium selenite) for 23 dogs*

| | |
|---|---|
| Range of dosage | 1.0–4.0 mgm. Se/kilogram |
| Lowest dosage to cause death in 24 hours | 1.5 mgm. Se/kilogram |
| Highest dosage which did not cause death in 24 hours | 2.25 mgm. Se/kilogram |
| Range of dosage killing 75 per cent in 24 hours | 1.50–2.00 mgm. Se/kilogram |

Discussion. The minimum fatal dose of selenium for the dog appears to be very nearly the same as that for the rat, when expressed on the same basis. We have preferred to use subcutaneous injection for the administration of the selenium because it gives the most uniform response. Intraperitoneal injections are sometimes accompanied by other complications which cause delayed death of the animal. Oral administration gives less consistent results depending upon the speed of assimilation and possibly the action of bacteria in the intestines. Animals are able to withstand somewhat larger doses when the selenium is administered orally or intraperitoneally and it would appear then that either the concentration or the toxicity of the selenium is somewhat reduced before it reaches the systemic circulation. Ultimately the oral method of administration will have to be investigated since animals which suffer from acute selenium poisoning under range conditions receive the selenium in their feed. The symptoms resulting from either method of administration are comparable but the minimum fatal dose is slightly lower when the selenium is injected subcutaneously.

The marked increase in the concentration of hemoglobin with the resulting increase in viscosity of the blood may in part explain the symptoms of suffocation. However, the observation by Potter and Elvehjem (16) that selenium inhibits the action of succinoxidase (succinic dehydrogenase) is another factor to be considered.

While the typical response shows a fall in inorganic phosphorus, the results are somewhat variable, especially when barbital and ether are used to depress the animal. In the five control dogs, which were treated in the same manner as those receiving selenium, we did not observe any significant lowering of the inorganic phosphorus of the blood as a result of veronal depression or ether anesthesia. However, we did observe a distinct drop in inorganic phosphorus in some of the dogs (which were to receive selenium) during the premedication or depression period. Subsequent work with more animals has shown that ether anesthesia and occasionally barbital depression may decrease the level of inorganic phosphorus in the blood. In each case, however, the selenium injection caused an additional fall. This was observed both in whole blood and in the plasma, which indicates that it is not merely a depletion effect brought about by the loss of fluid from the blood. Similar effects were noted for blood sugar and non-protein nitrogen.

The relationship between selenium and the reducing substances (glucose and ascorbic acid) is especially worthy of consideration since early workers (reviewed by Jones (5)) reported that there was a deposition of the red, amorphous form of metallic selenium in all tissues of animals which received injections of sodium selenite. After studying the reducing effects of various carbohydrates on sodium selenite Jones (5) concludes that glucosamine and fructose are capable of profuse reduction while glucose, arabinose, maltose and lactose are all capable of reducing the compound. He also reports that the amorphous form of metallic selenium is physiologically inert since animals which are able to survive such a dose of selenite could function normally so far as could be determined, while their tissues still contained the amorphous selenium.

The addition of ascorbic acid to the list of reducing agents (Levine (17)) offers a possible explanation of its beneficial effect. However, it may also exert a direct action on the capillaries, preventing the otherwise great fluid loss. It is also possible that some of the beneficial effects which resulted from injections of ascorbic acid may have been due in part to the phosphate buffer in which it was dissolved. Further work is necessary to clarify this point, as well as to study the effects of injections of glucose.

Svirbely (18) reported that rats suffering from acute selenium poisoning show marked decreases in the vitamin C content of their livers and adrenals. He also reported a protective action by glutathione in maintaining reduced ascorbic acid in the presence of selenium salts. Preliminary data from one dog (no. 13) show that the glutathione content of the blood parallels the general reduction and terminal increase in ascorbic acid (table 6). Perhaps the relationships between glutathione and ascorbic acid will explain why some dogs are able to mobilize large amounts of the latter into their blood stream and in some way exhibit increased resistance to the selenium. Reduced glutathione has been found to be effective in protecting rats against lethal doses of selenium in this laboratory (19). *It was, however, necessary to supply the glutathione in a ratio of ten moles* to one mole of selenite. More work will be needed to clarify the exact roles and interrelationships of glutathione, ascorbic acid, and blood sugar, but perhaps therein lies the explanation for the general decrease in ascorbic acid in the blood of most of the dogs, and marked increase in resistant dogs.

The importance of the loss of non-protein nitrogen cannot be stated at this time, but the decrease in concentration of this fraction is greater than might be explained on the basis of fluid loss.

Blood pressure measurements show a sharp terminal drop. Significant is the fact that there is no increase in blood pressure due to selenium injection, thus demonstrating that the loss of fluid from the blood is not due to a hydrostatic pressure but rather to some direct effect upon the blood vessels. This is confirmed by the observations at autopsy that the small blood vessels are greatly engorged and in some cases have a beaded appearance.

The concentration of the blood elements with the accompanying accumulation of fluid in the thoracic, and sometimes in the abdominal, cavity has been confirmed in this laboratory for the rat as well as the dog. Jones (5) was unable to observe these changes in his animals but does not state which animals were employed for such a study. Earlier in his report he mentions working with rats, rabbits, and cats but does not state whether one or all three species were used in his study of the effects upon the blood pressure and erythrocyte count. It is difficult then to determine where the discrepancy lies, but we have found the condition easily produced in the rat or dog.

The changes in the blood picture reported herein are typical for dogs suffering from acute selenium poisoning. The changes which are associated with chronic selenium poisoning have been described by Moxon[3] and Rhian[4].

## SUMMARY

(1) The minimal fatal dose of sodium selenite for the dog under barbital depression lies between 1.5 and 2.0 mgm. of selenium per kgm. of body weight when injected subcutaneously.

(2) Changes in the blood picture following such an injection are:

a. A marked increase in hemoglobin and hematocrit values, as much as 62%.

b. A decrease in inorganic phosphorus, non-protein nitrogen, ascorbic acid and blood sugar which is greater than might be explained by the fluid loss.

c. Preliminary data indicate a similar fall in glutathione concentration.

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[3] A. L. Moxon, M. S. Thesis, S. Dak. State College 1937 (On file in the S. Dak. State College Library.

[4] Morris Rhian, M. S. Thesis, S. Dak. State College 1939 (On file in the S. Dak. State College Library).

d. Blood pressure is not greatly affected until shortly before death, when it falls rapidly.

(3) Three relatively resistant dogs were encountered, two of which showed high values for plasma-ascorbic acid and the other showed high values for blood sugar.

(4) Efforts were made to increase the resistance of dogs by injecting ascorbic acid in M/15 phosphate buffer. Results indicate a favorable response when the selenium dosage was approximately 1 minimum fatal dose, since the usual increase in concentration of the formed elements was prevented or greatly reduced.

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# METABOLISM, TOXICITY AND MANNER OF ACTION OF GOLD COMPOUNDS USED IN THE TREATMENT OF ARTHRITIS

## IV. Studies of the Absorption, Distribution and Excretion of Gold Following the Intramuscular Injection of Gold Thioglucose and Gold Calcium Thiomalate

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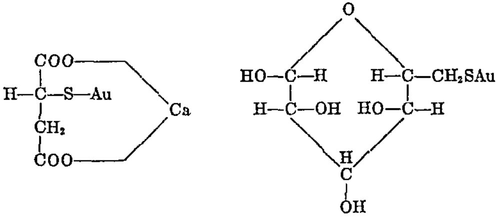
In a previous investigation (1) it was found that the absorption, distribution and excretion of gold after intramuscular administration to rats appeared to be related to the physical properties of the compound employed. The water soluble compounds were more rapidly absorbed than the insoluble colloidal preparations. Likewise, after the administration of the soluble preparations the greatest quantity of gold was found in the kidneys, which was in striking contrast with the large amount of gold in the liver following the injection of colloidal gold compounds. Furthermore, the soluble salts were excreted primarily through the kidneys and at a more rapid rate than the insoluble substances, which were excreted chiefly through the gastro-intestinal tract. Since this earlier study two additional gold compounds, gold calcium thiomalate and gold thioglucose have been made available. Because no data regarding either the absorption, distribution or excretion of the gold administered in these com-

Gold Compounds Studied



Gold Calcium Thiomalate[2]
(50 per cent gold)

Gold Thioglucose[3]
(50 per cent gold)

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[1] The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.

[2] Supplied by Merck & Co., Rahway, New Jersey.

[3] Supplied by Schering Corp., Bloomfield, New Jersey.

pounds are available it was thought advisable to investigate them from this standpoint and to compare the data with those previously obtained (1).

Procedure. Two groups of six white rats weighing 160 to 170 grams each were employed. Each animal received consecutive intramuscular injections of 1 mgm. of gold over a period of 14 days. One group received gold as gold calcium thiomalate and the other group as gold thioglucose. In both instances the compounds were administered in an oily suspension. Urine and feces were separated and collected quantitatively. On the fifteenth day the animals were decapitated, the tissues listed in table 1 were rapidly removed and analyzed for gold by the method of Block and Buchanan (2, 3). The urine and feces were pooled for the entire experimental period and analyzed separately.

TABLE 1

*Average gold content of various tissues, urine and feces following the intramuscular injection of several gold salts*

| TISSUE | GOLD CALCIUM THIOMALATE | | | GOLD THIOGLUCOSE | | |
|---|---|---|---|---|---|---|
| | *mgm.* | *per cent** | *mgm. per† gram* | *mgm.* | *per cent** | *mgm. per† gram* |
| Heart | 0.002 | 0.01 | 0.003 | 0.003 | 0.02 | 0.004 |
| Lung | 0.014 | 0.10 | 0.010 | 0.012 | 0.09 | 0.010 |
| Spleen | 0.029 | 0.21 | 0.018 | 0.051 | 0.37 | 0.034 |
| Liver | 0.215 | 1.54 | 0.022 | 0.432 | 3.09 | 0.049 |
| Kidney | 0.448 | 3.20 | 0.237 | 0.560 | 4.00 | 0.298 |
| Site of injection | 3.917 | 27.98 | 0.214 | 2.239 | 15.99 | 0.157 |
| Carcass | 1.869 | 13.35 | 0.011 | 2.641 | 18.86 | 0.015 |
| Urine | 1.522 | 10.87 | | 3.535 | 25.25 | |
| Feces | 3.995 | 28.54 | | 2.740 | 19.57 | |
| Total excretion‡ | 5.517 | 39.41 | | 6.275 | 44.82 | |
| Total | 12.011 | 85.7 | | 12.213 | 87.2 | |

* Values calculated as per cent of total gold injected.
† Values calculated as milligrams of gold per gram of tissue.
‡ Urine plus feces.

Results. In table 1 are listed the total amounts of gold found in various tissues, urine and feces as well as the amount of gold per gram of tissue. The total recovery of gold in the case of gold calcium thiomalate is 12.01 mgm. (85.7%) and in the case of gold thioglucose 12.21 mgm. (87.2%). The total amount excreted during the 14 day period is somewhat greater in the case of gold thioglucose. This is in direct correlation with the slower rate of absorption of gold calcium thiomalate as indicated by the greater amount of gold found at the site of injection in the animals treated with this salt. In view of our previous work (1) this finding was anticipated, since the gold calcium thiomalate is water-insoluble in contrast to the water-soluble gold thioglucose. We feel this difference is primarily associated with differences in physical properties of the two substances. In spite of the fact that both substances were administered in oily suspension, the difference in physical properties is manifested as soon as they come in contact with the body fluids. Likewise the greater excretion of the water soluble substance through the kidneys also confirms our previous findings. The quantity of gold is greater in the kidneys in both instances than in the liver. Thus after the injection of gold thioglucose in oil, gold is comparatively rapidly absorbed, is found in greatest amount in the kidneys and liver, and is excreted to a greater extent through the urinary tract. In all these respects it behaves similarly to other soluble gold compounds (administered in aqueous solution) and quantitatively seems comparable to gold sodium thiomalate. Gold calcium thiomalate behaves like other insoluble gold compounds in that it is more slowly absorbed and is excreted to a greater extent in the feces. But it is more like the soluble gold salts in that the greatest amount of gold is found in the kidneys. Thus gold calcium thiomalate stands intermediate in its properties between the colloidal insoluble gold compounds (colloidal gold sulfide) and the soluble salts such as gold sodium thiomalate (1).

## SUMMARY

(1) Following the intramuscular injection of oily suspensions of gold calcium thiomalate and gold thioglucose in the white rat the rate of absorption of gold is somewhat faster in the case of the latter compounds.

(2) Gold was found in larger amounts in the kidneys and liver than in other tissues studied.

(3) Excretion occurs through both the kidneys and gastro-intestinal tract. The chief route of excretion of the soluble gold thioglucose is the kidneys while gold is excreted primarily in the feces following administration of the insoluble gold calcium thiomalate.

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# THE TRYPANOCIDAL ACTION OF *3*-AMINO-*4*-HYDROXYPHENYL ARSENIOUS OXIDE ("MAPHARSEN") ADMINISTERED ORALLY WITH GLUTATHIONE

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Trivalent arsenicals of the arsenobenzol series are poorly absorbed from the gastrointestinal tract and possess little activity when administered by mouth. This is largely due to the fact that, with the exception of some interesting crystalloidal fractions recently isolated by Wright (1), the arsphenamines exhibit colloidal behavior, and arsphenamine itself is largely insoluble at the pH of the body. In view of the fact that *3*-amino-*4*-hydroxyphenyl arsenious oxide[1] is soluble and freely dialyzable at neutrality (2) experiments were carried out on its activity orally against *Trypanosome equiperdum* infections in mice. While many arsenic compounds have been investigated for therapeutic activity by mouth, interest has largely been centered upon pentavalent arsenicals.

Since it was anticipated that the arsenoxides would bring about local irritation of the intestinal canal, further experiments were conducted in which cysteine and glutathione were added to Aso prior to administration, for the purpose of decreasing the local and general toxicity. This was based upon the demonstration by Voegtlin, Dyer, and Leonard (3) that an excess of glutathione (10 moles) could antagonize the lethal effect of trivalent arsenious oxides administered intravenously as well as inhibit their local irritant action (4). Since an excess of glutathione was also shown to inhibit the therapeutic activity (5) of Aso intravenously, it remained to be determined to what extent this effect would occur when both substances were given by mouth.

Cohen, King, and Strangeways (6) have studied the trypanocidal action of a series of thioarsenites. Given intravenously to mice they found the di-glutathionyl derivative of *3*-amino-*4*-hydroxyphenyl arsenoxide (Aso) to be one-fifth as toxic and one-third as active as the parent compound. It is believed that in our experiments with oral administration we are dealing with a similar compound, since, as shown by the above authors, the condensation occurs readily when Aso is mixed with glutathione in aqueous solution.

Ewins and Everett (7) investigated a number of arsenic compounds with special reference to oral administration. Their most promising experimental preparations, in the arsinic acid series, failed to materialize when tested against human trypanosomiasis and syphilis. Some active oxides were also obtained, but their evidence indicates that the lowered toxicity was related to insolubility and poor absorption.

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[1] Subsequently referred to as Aso.

METHODS. Albino mice of 15 to 25 grams from an inbred strain grown at the Institute were employed. Intraperitoneal inoculations were made with *Trypanosome equiperdum* in sufficient quantity to produce death on the fourth or fifth day. Therapy was begun on the second day unless examination revealed an inadequate number of trypanosomes in the blood. Medication by mouth was administered through a small metal catheter attached to the tuberculin syringe. Freedom of the blood from trypanosomes for 30 days was taken as a criterion of cure.

ACUTE TOXICITY. The single maximum tolerated dose of Aso orally to mice was found to be 0.15 gram per kgm. This was not appreciably altered by the addition of 5 moles of partially neutralized cysteine. When mixed with 2 moles of glutathione, the tolerated dose was increased more than 3 times, to 0.5 gram per kgm. With 5 moles of glutathione the tolerated dose was greater than 1 gram per kgm.; since this represented 6.5 grams per kgm. of glutathione, higher doses at this level were not given.

The maximum tolerated dose of Aso intravenously in mice was 0.025 gram per kgm., which is approximately one-sixth the amount tolerated orally.

The oral toxicity of arsphenamine is low. Single doses of 1.5 grams perkgm. were tolerated by mice, while 2.0 grams per kgm. approached the L.D. 50. It is believed that the low toxicity represents poor absorption.

RESULTS IN TRYPANOSOME INFECTIONS IN MICE. The curative single dose of Aso administered orally was found to be 0.04 gram per kgm. This is approximately one-fourth the tolerated dose. The addition of 2 moles of glutathione did not appreciably alter the curative dose, so that under these circumstances with a tolerated dose of 0.5 gram per kgm. a curative ratio of 12 was obtained. The addition of 5 moles of glutathione caused some interference with the therapeutic activity of Aso, noted particularly at the lower dose levels and in the experiments with repeated administration; however, curative effects were obtained with doses of Aso of 0.06 to 0.08 gram per kgm. while the tolerated dose of Aso + 5 moles of glutathione was greater than 1 gram per kgm.

The curative dose of Aso administered intravenously under the conditions of our experiments was found to be 0.004 gram per kgm., approximately one-sixth of the amount tolerated by this route.

The ineffectiveness of arsphenamine by mouth is shown in that 0.25 gram per kgm. failed to sterilize any of the animals

Experiments with repeated administration of Aso were undertaken to compare the effects of a single curative dose with the same amount of drug given in divided doses over a period of days. It is seen that one-tenth the curative dose given 10 times during 5 consecutive days was no longer curative while one-fourth the curative dose given once daily for 4 days gave results comparable to those with a single dose.[2]

PRELIMINARY EXPERIMENT IN RABBIT SYPHILIS. Twenty-one rabbits with testicular chancres[3] were divided into three groups. Seven were treated orally with 10 mgm. per kgm. of Aso on 2 successive days. Seven were treated with this amount of Aso plus 5 moles of cysteine used in the absence of an adequate supply of glutathione. One of the animals in this second group died. Seven served as controls. Disappearance of the chancre and negative dark field examinations within 10 days were obtained in all treated animals and in none of the controls. Although sterilization was not established by gland transfer, these experiments demonstrate that Aso possesses treponemicidal activity when given by mouth.

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[2] Experiments with fractional doses by intravenous injection have been reported by Swinyard, Hirschfelder and Wright (This Journal 75: 367, 1942) since the submission of this paper. A comparison of results indicates that curative efficiency with fractional doses falls off even faster with intravenous adminstration than with oral.

TABLE 1

*Acute toxicity in mice*

| ASO | GMS. PER KGM. | ROUTE | NO. OF MICE | PER CENT MORTALITY |
|---|---|---|---|---|
| | 0.025 | oral | 5 | 0 |
| | 0.05 | " | 10 | 0 |
| | 0.1 | " | 10 | 0 |
| | 0.125 | " | 5 | 0 |
| | 0.15 | " | 10 | 10 |
| | 0.20 | " | 7 | 28.6 |
| | 0.25 | " | 20 | 70 |
| Aso + 2 moles glutathione | 0.25 | " | 10 | 0 |
| | 0.5 | " | 16 | 6.2 |
| | 0.625 | " | 5 | 40 |
| | 0.75 | " | 9 | 44.4 |
| Aso + 5 moles glutathione | 0.25 | " | 5 | 0 |
| | 0.5 | " | 8 | 0 |
| | 1.0 | " | 11 | 9 |
| Aso + 5 moles cysteine | 0.10 | " | 5 | 0 |
| | 0.125 | " | 10 | 20 |
| | 0.25 | " | 10 | 40 |
| | 0.5 | " | 5 | 100 |
| Aso | 0.015 | i.v. | 5 | 0 |
| | 0.020 | " | 5 | 0 |
| | 0.025 | " | 5 | 0 |
| | 0.030 | " | 5 | 100 |
| Arsphenamine | 1.0 | oral | 15 | 0 |
| | 1.5 | " | 10 | 0 |
| | 2.0 | " | 10 | 40 |

Gruhzit (8) has reported Aso ineffective orally in rabbit syphilis when 1 mgm. per kgm. was administered daily for 16 days. This is consistent with our

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[3] These were obtained from Mr. T. F. Probey of the Division of Biologics Control of this Institute, who kindly cooperated in carrying out dark field examinations.

## TABLE 2

*Trypanosome therapy in mice*

| ORAL THERAPY | NO. OF MICE | BLOOD NEG. | AVERAGE SURVIVAL | PER CENT SURVIVAL |
|---|---|---|---|---|
| | | *days** | *days** | |
| Aso | | | | |
| 0.005 gm. per kilogram | 7 | 7 | 13 | 0 |
| 0.0125 " " " | 8 | | 14 | 0 |
| 0.02 " " " | 10 | 10.2 | 12.6 | 10 |
| 0.025 " " " | 8 | 30 | 30 | 100 |
| 0.04 " " " | 8 | 25 | 25.5 | 76 |
| 0.04 " " " | 9 | 30 | 30 | 100 |
| 0.04 " " " | 9 | 28 | 28.3 | 78 |
| 0.05 " " " | 9 | | 26 | 89 |
| 0.05 " " " | 9 | 30 | 30 | 100 |
| 0.06 " " " | 9 | 30 | 30 | 100 |
| 0.06 " " " | 10 | 30 | 30 | 100 |
| Aso | | | | |
| 0.25 + 5 moles cysteine | 9 | | 15 | 11 |
| Aso | | | | |
| 0.04 + 1 mole glutathione | 8 | 30 | 30 | 100 |
| 0.02 + 2 moles " | 10 | 22.1 | 23 | 60 |
| 0.04 + 2 moles " | 9 | 28.3 | 29 | 89 |
| 0.04 + 2 moles " | 10 | 26.7 | 27.6 | 70 |
| 0.06 + 2 moles " | 9 | 30 | 30 | 100 |
| Aso | | | | |
| 0.025 + 5 moles glutathione | 8 | 16.4 | 19.4 | 37 |
| 0.05 + 5 moles " | 10 | 19.2 | 23.7 | 50 |
| 0.06 + 5 moles " | 8 | 30 | 30 | 100 |
| 0.08 + 5 moles " | 8 | 30 | 30 | 100 |
| Arsphenamine | | | | |
| 0.250 gm. per kilogram | 9 | | 8.8 | 0 |
| 0.1 " " " | 10 | 0 | 8.7 | 0 |
| 0.05 " " " | 10 | 0 | 2.5 | 0 |
| 0.0025 " " " | 10 | 0 | 2.0 | 0 |
| Intravenous therapy | | | | |
| Aso | | | | |
| 0.002 gm. per kilogram | 10 | 15.3 | 17.7 | 30 |
| 0.004 " " " | 10 | 28.1 | 28.6 | 90 |
| Repeated therapy | | | | |
| Aso | | | | |
| 0.0025 B.D. × 5 days | 10 | | 4.4 | 0 |
| 0.00375 B.D. × 5 days | 10 | | 13.1 | 10 |
| 0.005 B.D. × 5 days | 9 | | 22.7 | 44.4 |
| 0.0125 B.D. × 4 days | 6 | 28 | 28 | 82.5 |
| 0.0125 B.D. × 4 days + Glutathione—5 moles | 6 | 21 | 22.5 | 50 |
| Arsphenamine | | | | |
| 0.05 B.D. × 5 days | 9 | | 17.5 | 33.3 |

results in trypanosome infections where a small fraction of the curative dose was administered repeatedly over a period of time.

## SUMMARY

*3*-Amino-*4*-hydroxyphenyl arsenious oxide administered by mouth possesses curative action against *Trypanosome equiperdum* infections in mice.

The addition of 2 moles of glutathione decreased toxicity without affecting therapeutic activity, giving a therapeutic ratio that compares favorably with intravenous administration.

Preliminary experiments indicate some therapeutic action in rabbit syphilis following oral therapy.

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# ON THE NATURE OF THE EMETIC ACTION OF THE DIGITALIS BODIES AND RELATED COMPOUNDS[1]

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The emetic action of the digitalis bodies, as demonstrated by Hatcher and Eggleston (1), does not depend upon reflexes originating in the gastro-intestinal tract. The conclusion drawn by Eggleston and Hatcher (2) that digitalis produces nausea and vomiting as a result of direct action on the medullary vomiting center is not supported by the observations of Hatcher and Weiss (3, 4) that direct application of digitalis bodies to the center does not induce vomiting. After interruption of the afferent nerve pathways which travel from the heart to the medulla Hatcher and Weiss (3, 5) failed to obtain the nausea and vomiting response in most of the animals tested. They therefore concluded (3, 5, 6) that the emetic action of digitalis bodies is dependent upon reflexes originating as a result of direct action of the compounds on the heart. The experiments of Hatcher and French (7), in which nicotine abolished the emetic action of strophanthidin, were presented as evidence that this action has its origin at the peripheral ends of cardiac afferent nerve fibers. A cardiac origin of the emetic action of digitalis bodies and related compounds seems highly unlikely in view of the observations of Dresbach and Waddell (8, 9, 10) which indicate that emesis in response to *k*-strophanthidin is not abolished by vagotomy, stellate ganglionectomy, thoracic sympathectomy and spinal cord section at the seventh cervical level. The recent preliminary report by Dresbach (11) has further substantiated his earlier observations.

It seemed to us that further study of the problem by means of the denervated heart preparation might assist in clarifying the picture. In using this preparation one is confronted with the fact that while vagotomy is a certain means of abolishing the parasympathetic innervation of the heart the usual methods of sympathetic denervation of this organ are less certain. Accordingly the following experiments are designed to test the ability of digitalis bodies and related compounds to produce nausea and vomiting in the dog whose heart is almost certainly completely denervated as judged by its response to several physiological tests.

METHOD. Normal well-nourished dogs were trained to lie quietly while connected in the usual manner to an electrocardiograph and while submitting to venipuncture. At intervals of two or three weeks the following operations were performed under aseptic technique. Removal (by cautery) of the adrenal medulla on one side; a similar operation on the opposite side; removal of the stellate and upper 6 thoracic sympathetic ganglia on one side; a similar sympathectomy on the other side; bilateral vagotomy high in the neck. Following section of the vagi the animals were taken off food and were given daily intra-

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[1] *Aided by a grant from the John and Mary R. Markle Foundation.*

results in trypanosome infections where a small fraction of the curative dose was administered repeatedly over a period of time.

## SUMMARY

*3*-Amino-*4*-hydroxyphenyl arsenious oxide administered by mouth possesses curative action against *Trypanosome equiperdum* infections in mice.

The addition of 2 moles of glutathione decreased toxicity without affecting therapeutic activity, giving a therapeutic ratio that compares favorably with intravenous administration.

Preliminary experiments indicate some therapeutic action in rabbit syphilis following oral therapy.

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# ON THE NATURE OF THE EMETIC ACTION OF THE DIGITALIS BODIES AND RELATED COMPOUNDS[1]

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The emetic action of the digitalis bodies, as demonstrated by Hatcher and Eggleston (1), does not depend upon reflexes originating in the gastro-intestinal tract. The conclusion drawn by Eggleston and Hatcher (2) that digitalis produces nausea and vomiting as a result of direct action on the medullary vomiting center is not supported by the observations of Hatcher and Weiss (3,4) that direct application of digitalis bodies to the center does not induce vomiting. After interruption of the afferent nerve pathways which travel from the heart to the medulla Hatcher and Weiss (3, 5) failed to obtain the nausea and vomiting response in most of the animals tested. They therefore concluded (3, 5, 6) that the emetic action of digitalis bodies is dependent upon reflexes originating as a result of direct action of the compounds on the heart. The experiments of Hatcher and French (7), in which nicotine abolished the emetic action of strophanthidin, were presented as evidence that this action has its origin at the peripheral ends of cardiac afferent nerve fibers. A cardiac origin of the emetic action of digitalis bodies and related compounds seems highly unlikely in view of the observations of Dresbach and Waddell (8, 9, 10) which indicate that emesis in response to *k*-strophanthidin is not abolished by vagotomy, stellate ganglionectomy, thoracic sympathectomy and spinal cord section at the seventh cervical level. The recent preliminary report by Dresbach (11) has further substantiated his earlier observations.

It seemed to us that further study of the problem by means of the denervated heart preparation might assist in clarifying the picture. In using this preparation one is confronted with the fact that while vagotomy is a certain means of abolishing the parasympathetic innervation of the heart the usual methods of sympathetic denervation of this organ are less certain. Accordingly the following experiments are designed to test the ability of digitalis bodies and related compounds to produce nausea and vomiting in the dog whose heart is almost certainly completely denervated as judged by its response to several physiological tests.

METHOD. Normal well-nourished dogs were trained to lie quietly while connected in the usual manner to an electrocardiograph and while submitting to venipuncture. At intervals of two or three weeks the following operations were performed under aseptic technique: Removal (by cautery) of the adrenal medulla on one side; a similar operation on the opposite side; removal of the stellate and upper 6 thoracic sympathetic ganglia on one side, a similar sympathectomy on the other side; bilateral vagotomy high in the neck. Following section of the vagi the animals were taken off food and were given daily intra-

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[1] Aided by a grant from the John and Mary R. Markle Foundation.

venous injection of 10% glucose in amounts sufficient to keep them in good condition for several days.

In two additional experiments the animals were prepared according to the following procedure: Under cyclopropane anesthesia the spinal cord was transected at the sixth cervical level and pithed from there caudally through the remainder of its length. The vagi were cut high in the neck. Within a half hour after the withdrawal of the anesthetic the animals were considered ready for use.

The four emetic agents employed were Strophosid,[2] Cedilanid,[3] Digilanid,[4] and Digiglusin.[5] Each was administered in a dose approximately equal to 1 cat unit per 10 pounds of body weight. In all but one experiment the compounds were administered intravenously. Nausea and vomiting usually follow within 10 minutes after injection of this dosage in the normal dog. If emesis failed to occur within 10 or 15 minutes, a second, and in some cases a third smaller dose was administered.

All animals were tested for completeness of the denervation of the heart by following the rate electrocardiographically before, during and after the intravenous injection of the pressor compound pitressin in a dose of 1 pressor unit, and the depressor compound nitroglycerine (U. S. P. spirits diluted 25 times) in a dose of 1 cc. The ability of these compounds to produce changes in blood pressure of the unanesthetized, spinal cord-pithed, vagotomized animal was determined.

RESULTS. *1. Dogs whose hearts were denervated by bilateral removal of the stellate and upper 6 thoracic sympathetic ganglia and section of both vagi.* As indicated in table 1, each of the 3 dogs with denervated hearts vomited in response to digitalis bodies or *k*-strophanthoside[2] administered intravenously. It will be noted in experiments 1 and 4 that 2 additional doses of the emetic agent were given before emesis occurred. In experiments 3, and 5 to 9 inclusive, vomiting in each case followed within 6 to 8 minutes after the intravenous injection of 1 cat unit per 10 pounds of body weight. In experiment 10 the injection was made subcutaneously and as a result the emesis was delayed.

The results of tests for the completeness of the denervation of the hearts of the 3 dogs of this group are given in table 2. It will be noted that neither the pressor compound pitressin nor the depressor compound nitroglycerine were capable of inducing more than slight changes in the rate of the heart of these animals. The ability of these compounds to produce changes in the level of blood pressure was studied in the spinal cord-pithed, vagotomized animal without anesthesia. One pressor unit of pitressin, intravenously, caused a prompt rise of blood pressure to a level 90 mm. of mercury above the pre-injection reading. One cc. of the nitroglycerine solution caused a fall of 50 mm. of mercury in the same animal.

*2. Dogs whose spinal cord was sectioned at the sixth cervical level and pithed from that point caudally throughout the remainder of its length, and whose vagi were cut high in the neck.* The results obtained on these animals are included in table 1,

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[2] Strophosid is a brand of K-Strophanthoside distributed by Sandoz Chemical Works, New York.

[3] Cedilanid is a brand of Lanatoside C produced by Sandoz Chemical Works, New York.

[4] Digilanid is a brand of Lanatosides A, B, and C produced by Sandoz Chemical Works, New York.

[5] Digiglusin is a brand of a mixture of digitalis glucosides produced by Eli Lilly and Company, Indianapolis, U. S. A.

TABLE 1

| EXPT. NO. | DATE | DOG NO. | WT. | CONDITION OF ANIMAL* | PREPARATION USED† |  |  | RESULT |  |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | Name of Compound | Dose | Time | Type of Response | Time |
|  |  |  | *pounds* |  |  |  |  |  |  |
| 1 | 4-21 | 2 | 33 | D.H. | Digilanid | 2.4 | 3:57 |  |  |
|  |  |  |  |  | " | 1.2 | 4:10 |  |  |
|  |  |  |  |  | " | 1.2 | 4:20 | Emesis | 4:24 |
| 2 | 4-29 | 1 | 24 | D.H. | Digilanid | 2.4 | 2:20 | Emesis | 2:46 |
| 3 | 4-29 | 3 | 24 | D.H. | Cedilanid | 2.4 | 3:52 | Emesis | 4:00 |
| 4 | 5-1 | 2 | 33 | D.H. | Cedilanid | 3.2 | 3:55 |  |  |
|  |  |  |  |  | " | 0.8 | 4:25 |  |  |
|  |  |  |  |  | " | 0.8 | 4:28 | Emesis | 4:36 |
| 5 | 5-7 | 1 | 24 | D.H. | Cedilanid | 2.4 | 10:52 | Emesis | 11:00 |
| 6 | 5-7 | 3 | 20 | D.H. | Cedilanid | 2.0 | 11:40 | Emesis | 11:48 |
| 7 | 5-8 | 1 | 24 | D.H. | Strophosid | 2.0 | 2:00 | Emesis | 2:08 |
| 8 | 5-8 | 3 | 20 | D.H. | Strophosid | 2.0 | 2:15 | Emesis | 2:21 |
| 9 | 5-12 | 1 | 24 | D.H. | Digiglusin | 2.0 | 2:33 | Emesis | 2:41 |
| 10 | 5-12 | 3 | 20 | D.H. | Digiglusin‡ | 2.0 | 2:36 | Salivation and licking of lips | 3:00 |
|  |  |  |  |  | Digiglusin‡ | 1.0 | 3:15 | Emesis | 3:45 |
| 11 | 5-15 | 4 | 24 | S.C. pithed | Cedilanid | 2.4 | 10:16 | Salivation and licking lips | 10:25 |
|  |  |  |  |  |  |  |  | Typical signs of emesis response | 10:30 |
| 12 | 6-6 | 5 | 16 | S.C. pithed | Cedilanid | 1.6 | 10:30 | Typical signs of emesis response | 10:43 |

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* Condition of the animal; D.H. refers to the dogs whose hearts have been denervated by section of both vagi high in the neck and removal of the stellate and upper 6 thoracic sympathetic ganglia on both sides. S.C. pithed refers to the dogs whose spinal cord was sectioned at the sixth cervical level and pithed from there caudally through the remainder of its length, and whose vagi were cut high in the neck.

† See footnotes 2, 3, 4, 5 for description of the compounds used. Dosage is given in cat units.

‡ Administered subcutaneously. All others were administered intravenously.

experiments 11 and 12. It will be noted in each case that 1 cat unit of Cedilanid produced emesis in less than 15 minutes. It must be recognized, of course, that contraction of the abdominal muscles is impossible in these animals and that other criteria of an emetic action must be looked for. Among the signs of an emetic response observed in these animals were profuse salivation, licking of the lips, repeated swallowing movements, wide opening of the mouth with gasping movements and forceful contractions of the diaphragm. These findings were accepted as unmistakable signs of an emetic action.

TABLE 2

| DOG NO. | CONDITION OF ANIMAL* | COMPOUND† | BEFORE INJECT. | 15 SEC. PERIODS AFTER INJECT. | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 |
| 1 | D.H. | Nitroglycerine | 132 | 128 | 134 | 134 | 130 | 130 |
| 2 | D.H. | Nitroglycerine | 152 | 152 | 158 | 158 | 158 | 160 |
| 3 | D.H. | Nitroglycerine | 102 | 102 | 106 | 108 | 110 | 108 |
| 1 | D.H. | Pitressin | 144 | 144 | 144 | 144 | 144 | |
| 2 | D.H. | Pitressin | 126 | 128 | 124 | 124 | 124 | |
| 3 | D.H. | Pitressin | 158 | 160 | 160 | 158 | 160 | |
| 4 | S.C. pithed | Nitroglycerine | 112 | 112 | 114 | 114 | 114 | 112 |

All of the data are presented as heart beats per minute calculated from the rate during 15 second intervals before and after injection.

* D.H. refers to denervated heart. S.C. pithed refers to spinal cord-pithed, vagotomized dogs. See Table I for body weights of these dogs.

† Nitroglycerine was given in doses of 1 cc. of 1 to 25 dilution of U. S. P. spirits. Pitressin was given in doses of 1 pressor unit.

Discussion. The experiments designed to test for the completeness of the denervation of the hearts of dogs 1, 2 and 3 failed to show more than slight changes in heart rate in response to either the depressor compound nitroglycerine or the pressor compound pitressin. As indicated in the results the doses of these compounds were sufficient to produce marked changes in blood pressure in the unanesthetized, spinal cord-pithed, vagotomized dog. Therefore it may reasonably be judged that the hearts of dogs 1, 2 and 3 were completely denervated. There can be little doubt about the completeness of the denervation of the hearts of the 2 spinal cord-pithed, vagotomized animals.

In the case of the depressor compound nitroglycerine, a slight increase in heart rate developed only after an interval of 15 seconds. This response is readily explained on the basis of humoral mechanisms involving reflexly liberated sympathin. The early reflex tachycardia which develops in the normal animal within a few seconds after the injection of nitroglycerine was entirely lacking in our dogs with denervated hearts.

Of interest is the fact that none of the 3 denervated heart dogs developed a slowing of the heart in response to pitressin. In another series of experiments, however, we have observed an occasional inhibition of the denervated heart in response to pitressin, a finding which is of interest in the light of the reports of Gruber and Kountz (12, 13) that pitressin inhibits the hearts of vagotomized and atropinized animals. These workers attributed the inhibition of the heart of such animals to coronary vasoconstriction and direct cardiac effects of the compound.

In all of the 12 experiments on 3 chronic denervated heart dogs and 2 spinal cord-pithed, vagotomized dogs an emetic response to digitalis bodies or related compounds was observed. The time interval between injection and response in most cases was well within the limits commonly accepted for normal dogs. These results are in agreement with those of Dresbach and Waddell (8, 9, 10) and Dresbach (11) who found that denervation of the hearts of cats and dogs does not abolish the emetic action of strophanthidin, ouabain, thevitin, *K*-strophanthosid and lanatosides A, B and C. To the extent that they eliminate the heart as the seat of origin of the emetic response they also are in agreement with the findings of Hanzlik and Wood (14) in their study of the mechanism and site of origin of digitalis emesis in pigeons. While section of the vagi abolished the response in the pigeon, section of only the cardiac branches of these nerves failed to do so. The conclusion that in the pigeon non-cardiac vagal afferents are essential to the emetic action of digitalis would seem to be justified, although it is not supported by the results obtained on cats and dogs as already outlined.

The question of the site of origin of the emesis response to digitalis and related compounds is not readily answered. The failure to elicit vomiting in response to the local application of digitalis bodies to the floor of the medulla of anesthetized animals (Hatcher and Weiss, 3, 4) obviously does not offer convincing evidence against a direct stimulatory influence of these compounds on the vomiting center. On the other hand the results of the experiments which have eliminated the gastro-intestinal tract (Hatcher and Eggleston, 1) and the liver (Dresbach, 15) as well as the heart (Dresbach *et al.*, 8, 9, 10, 11; and those presented in this report) do not prove that the emesis response is due to a direct central action of the compounds. The evidence presented in the preliminary report of Dresbach (11) and that offered in the present paper rule out the possibility that afferents from most of the other viscera including those of the pelvis are essential to the response. The possibility remains however that afferents in the phrenic nerves (16) may be concerned. Proof one way or the other on this point would have little to offer in establishing the site of the emetic action of the digitalis bodies as being central or peripheral. The question can be settled only by experiments designed to remove the medullary vomiting center from all afferent nerve connections and to permit observations to be made without anesthesia and while the animal is in good general condition. It would seem that the problem will not be solved readily because of the difficulty of satisfying such requirements.

It is clear that the results of the experiments on the chronically denervated heart dogs prove that the emetic action of the digitalis bodies and related compounds used in these experiments does not depend on afferent nerve fibers from experiments 11 and 12. It will be noted in each case that 1 cat unit of Cedilanid produced emesis in less than 15 minutes. It must be recognized, of course, that contraction of the abdominal muscles is impossible in these animals and that other criteria of an emetic action must be looked for. Among the signs of an emetic response observed in these animals were profuse salivation, licking of the lips, repeated swallowing movements, wide opening of the mouth with gasping movements and forceful contractions of the diaphragm. These findings were accepted as unmistakable signs of an emetic action.

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| 3 | D.H. | Nitroglycerine | 102 | 102 | 106 | 108 | 110 | 108 |
| 1 | D.H. | Pitressin | 144 | 144 | 144 | 144 | 144 | |
| 2 | D.H. | Pitressin | 126 | 128 | 124 | 124 | 124 | |
| 3 | D.H. | Pitressin | 158 | 160 | 160 | 158 | 160 | |
| 4 | S.C. pithed | Nitroglycerine | 112 | 112 | 114 | 114 | 114 | 112 |

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# ACTION OF BENZEDRINE SULFATE ON THE EXCITABILITY OF NERVE, MUSCLE AND HEART IN THE FROG (*CALYPTOCEPHALUS GAYI*)

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In 1938 Meidinger (1) found that addition of benzedrine to isolated nerves immersed in physiologic solution caused an enormous increase in the rheobase without change in chronaxie. Since the action of benzedrine is not limited to the nervous system, the present investigation was undertaken to determine its effect on cardiac and skeletal muscle as well as isolated nerves.

METHODS. The estimations were made with a chronaximeter with condenser discharges according to the principle of Lapicque (1926). The nerve-muscle (sciatic-gastrocnemius) preparation was placed in a moist chamber made of solid paraffin. This was divided into two sections, one of which contained the muscle moistened with cotton soaked in Ringer's solution, and the other the nerve. The two compartments were connected by a groove which permitted passage of the nerve. A seal was effected with neutral petrolatum. The nerve was held on two non-polarizable Ag-AgCl electrodes 20 mm. apart. Tests were made by placing the physiologic solution containing benzedrine in the nerve compartment and allowing the fluid to remain for a specified period. The fluid then was aspirated, the compartment carefully dried, and the nerve again held on the electrodes. A two-minute interval was allowed to elapse before measurements were made. The test applied was minimal contraction of the gastrocnemius muscle, which can be estimated perfectly with adequate illumination of the muscle surface. Measurements were made in the following order: *1*. Rheobase. *2*. Chronaxie. *3*. Rheobase control. *4*. Estimation of voltage quotient Q according to the technic described by Günther (2) The rheobase condenser (10 microfarads) discharges through the shunt formed by a resistance of 4000 ohms and two continuous parallel resistances of 10,000 and 11,000 ohms; the object is in series with the latter. To obtain an exponential increase in the stimulating current, a 2-microfarad condenser is placed parallel to the last two resistances. The numerator (*Vc*) corresponds to the threshold with current of the exponential elevation, and the denominator (*Vr*) is the value of the rheobase. The quotient *Q* is an index of the speed with which "accommodation" takes place. The average normal values found for Q with this procedure were 2.7 for the sciatic nerve and 1 04 for the frog heart.

To study the excitability of the isolated gastrocnemius, the femur was clamped securely and the distal end of the muscle was connected with an isometric lever arranged to record its contractions on the smoked paper of a kymograph. The electrodes were two Ag-AgCl wires, 0.5 mm. in diameter, attached to the ends of the muscle. To study the action of benzedrine the muscle was immersed in a solution of this substance. The course of a complete estimation with a record of the contractions is given in Fig. 1 (Rheobase), Fig. 2 (chronaxie), and Fig. 3 (voltage quotient). Estimations also were made of the excitability of the heart, arrested by a Stannius ligature and subjected to unipolar stimulation according to the technic of Cicardo and Marenzi (3). Two electrodes were used, one a silver plate placed in the animal's mouth, the other an Ag-AgCl wire fastened at the apex of the heart.

RESULTS. A. *Neuromuscular excitability of frogs poisoned by benzedrine sulfate.* The injection of 150–600 mgm. per kgm. of benzedrine sulfate into the ventral lymph sac of the frog caused progressive abolition of reflexes. An hour later the animal was decapitated (except the frog in Experiment 2, table 1, which was injected with 600 mgm. per kgm. and died in less than an hour), and sciatic-gastrocnemius preparations were made. The results are shown in table 1.

It is interesting to note that the rheobase was unchanged. The chronaxie, however, was greatly increased except in Experiments 5 and 6, where these values were normal. The voltage quotient fell sharply except in Experiments 3 and 6. The increase in the chronaxie apparently corresponds to a decrease in excitability. To this we must add a diminution in the rate of nerve accommodation in the poisoned animal as deduced from the reduction of $Q$.

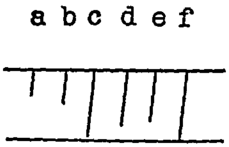

FIG. 1. ESTIMATION OF THE RHEOBASE OF THE GASTROCNEMIUS IN A FROG POISONED FOR ONE HOUR WITH 1% BENZEDRINE.

Voltage: a) 5.1; b) 5.4; c) 5.7; d) 5.85; e) 5.9; f) 6.0 = rheobase.

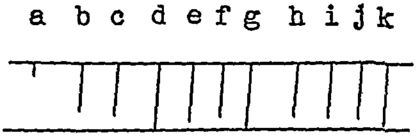

FIG. 2. ESTIMATION OF THE CHRONAXIE IN THE SAME MUSCLE.

Microfarads: a) 0.100; b) 0.110; c) 0.120; d) 0.130; e) 0.120; f) 0.122; g) 0.124; h) 0.124; i) 0.125; j) 0.126; k) 0.127 = chronaxial capacitance.

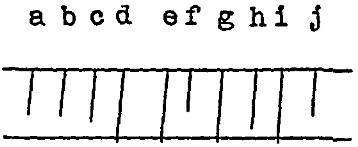

FIG. 3. CONTROL OF THE RHEOBASE Vr AND ESTIMATION OF THE THRESHOLD WITH EXPONENTIAL CURRENT Vc.

a) 5.85; b) 5.90; c) 6.00; d) 6.10 = Vr; e) 12.0; f) 11.1; g) 11.4; h) 11.2; i) 11.25 = Vc; j) 11.1 = control.

B. *Action of benzedrine sulfate on the isolated nerve.* When the nerve was immersed several times in a 0.5% solution of benzedrine, there was a progressive increase in the rheobase up to the point of inexcitability in the course of one or two hours. The chronaxie decreased slightly, while the quotient $Q$ increased progressively. This indicates an increase in the rate of nervous accommodation.

In table 2 are summarized all the results obtained with different concentrations of benzedrine. The rheobase of the nerves immersed in Ringer's solution for half an hour increased 50%, but when the nerves were placed in a solution of 0.5% benzedrine there was an increase of 200–300%. The chronaxie of the nerves decreased slightly or remained unchanged, whether in Ringer's solution or benzedrine. In either case the quotient $Q$ showed no deviation from its typical form. In Experiments 8, 9, and 10 the animals previously had been poisoned by benzedrine for 60 to 90 minutes, after which the nerves were placed in a solution of 0.5% or 1.0% benzedrine. In this case also there was an increase in the rheobase (60–180%) and a relatively slight decrease in the chronaxie, while the voltage quotient $Q$ was unchanged. To summarize, it may be said that a nerve immersed in benzedrine solution shows a great increase in the rheobase with maintenance of the chronaxial values, which is in agreement with the results obtained by Meidinger (1). In animals previously poisoned by benzedrine the increase in the rheobase was less marked.

TABLE 1

*Nerve-muscle (sciatic-gastrocnemius) excitability of frogs poisoned with benzedrine sulfate and killed one hour after injection in the ventral lymph sac*

| EXPERIMENT NUMBER | BENZEDRINE (mgm per kgm) | NEUROMUSCULAR PREPARATION | RHEOBASE (millivolts) | CHRONAXIE (sigmas) | $Q = Vc/Vr$ |
|---|---|---|---|---|---|
| 1 | 300 | right | 240 | 1.88 | 1.75 |
| | | left | 190 | 1.64 | 1.42 |
| 2 | 600 | right | 150 | 0.56 | 1.33 |
| | | left | 165 | 0.56 | 1.28 |
| 3 | 250 | right | 285 | 1.76 | 3.2 |
| | | left | 270 | 1.52 | 4.5 |
| 4 | 250 | right | 225 | 0.48 | 1.78 |
| | | left | 210 | 0.48 | 1.75 |
| 5 | 300 | right | 240 | 0.34 | 1.78 |
| 6 | 150 | right | 150 | 0.30 | 2.55 |
| | | left | 220 | 0.33 | 2.13 |
| 7 | Control | right | 220 | 0.30 | 2.6 |
| | | left | 330 | 0.27 | 2.7 |

C. *Action of benzedrine on the isolated gastrocnemius muscle.* In order to study the effect of this substance, not only on the isolated nerve but also on the entire neuromuscular system, the muscle was immersed in a physiologic solution containing a certain concentration of benzedrine. The results are summarized in table 3. The controls in Ringer's solution showed an increase of 40–70% in the rheobase and 10–90% in the chronaxie, while $Q$ decreased in every case. With benzedrine there was always an enormous increase in the rheobase. The remarkably long chronaxie is attributable in part to the great degree of separation of the stimulating electrodes. Another factor is that the values obtained by this test do not represent excitation of the more sensitive fibers, but correspond with the average of a certain number of fibers capable of developing the tension previously fixed as the threshold. More dilute solutions of benzedrine (0.1 and 0.5%) caused, except in Experiment 4, a 50% decrease in the chronaxie. In 1% solution benzedrine also caused a diminution in the chronaxie. A diphasic

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*Right neuromuscular (sciatic-gastrocnemius) preparation from a frog decapitated at 2:50 p.m. Nerve and muscle in moist chamber. Bipolar stimulation of nerve*

| TIME | RHEOBASE | CAPACITY | CHRONAXIE | Vc/Vr | Q = Vc/Vr | NERVE IMMERSED IN 0.5 PER CENT BENZEDRINE SOLUTION |
|---|---|---|---|---|---|---|
| | *millivolts* | *microfarads* | *sigmas* | *millivolts* | | *minutes* |
| 3:35 | 120 | 0.088 | 0.342 | 285/140 | 2.03 | |
| 3:42 | | | | | | 30 |
| 4:25 | 250 | 0.087 | 0.348 | 780/285 | 2.74 | |
| 4:26 | | | | | | 30 |
| 5:08 | 570 | 0.066 | 0.264 | 2400/750 | 3.20 | |
| 5:10 | | | | | | 40 |
| 6:00 | Inexcitable | | | | | |

TABLE 2

*Action of benzedrine on the excitability of nerve-muscle preparation in the frog*

| EXPERIMENT NUMBER | DURATION OF SUBMERSION OF NERVE | SOLUTION | RHEOBASE (MILLIVOLTS) | | CHRONAXIE (SIGMAS) | | Q = Vc/Vr | | REMARKS |
|---|---|---|---|---|---|---|---|---|---|
| | | | *Before* | *After* | *Before* | *After* | *Before* | *After* | |
| | *minutes* | | | | | | | | |
| 1 | 90 | Ringer | 180 | 300 | 0.38 | 0.31 | 1.67 | 1.70 | |
| 2 | 30 | Ringer | 220 | 340 | 0.30 | 0.27 | 2.6 | 2.7 | |
| 3 | 30 | Ringer | 300 | 420 | 0.37 | 0.36 | 1.9 | 1.7 | |
| 4 | 15 | Benz. 0.5% | 105 | 300 | 0.30 | 0.27 | 2.72 | 2.4 | |
| 5 | 30 | Benz. 0.5% | 120 | 250 | 0.34 | 0.34 | 2.03 | 2.74 | |
| 6 | 30 | Benz. 0.1% | 150 | 255 | 0.30 | 0.30 | 2.84 | 2.30 | |
| 7 | 30 | Benz. 0.5% | 160 | 465 | 0.36 | 0.30 | 2.1 | 2.1 | |
| 8 | 30 | Benz. 0.5% | 150 | 420 | 0.33 | 0.26 | 2.9 | 2.93 | Frog poisoned by 300 mgm. per kgm. (90 minutes) |
| 9 | 15 | Benz. 1.0% | 240 | 390 | 0.36 | 0.30 | 1.95 | 2.2 | Frog poisoned by 300 mgm. per kgm. (60 minutes). Right preparation |
| 10 | 15 | Benz. 1.0% | 250 | 480 | 0.29 | 0.28 | 2.12 | 2.05 | Left preparation from above frog |

action was apparent, however, for prolonged immersion in benzedrine resulted in a considerable increase in the chronaxie (see particularly Experiments 8 and 10). With dilute solutions of benzedrine the quotient $Q$ increased, while with more concentrated solutions $Q$ varied simultaneously but inversely with the chronaxie. Boyd (4) reported that benzedrine decreased the intensity of contractions of the gastrocnemius muscle stimulated by galvanic current. This is in agreement with the reduction in excitability described above.

TABLE 3

*Action of benzedrine sulfate on the excitability of the gastrocnemius muscle of the frog*

| EXPERIMENT NUMBER | RHEOBASE (VOLTS) | | CHRONAXIE (SIGMAS) | | $Q = Vc/Vr$ | | SOLUTION | DURATION OF IMMERSION |
|---|---|---|---|---|---|---|---|---|
| | *Before* | *After* | *Before* | *After* | *Before* | *After* | | |
| | | | | | | | | *minutes* |
| 1 | 1.4 | 2.35 | 0.28 | 0.53 | 2.3 | 1.8 | Ringer | 30 |
| 2 | 3.3 | 5.7 | 0.48 | 0.56 | 1.94 | 1.70 | Ringer | 60 |
| 3 | 2.4 | 3.45 | 0.30 | 0.33 | 2.16 | 2.0 | Ringer | 60 |
| 4 | 1.95 | 5.45 | 1.0 | 3.2 | 1.70 | 1.86 | Benz. 0.1% | 60 |
| 5 | 2.4 | 4.6 | 0.72 | 0.37 | 1.16 | 1.26 | Benz. 0.1% | 60 |
| 6 | 1.8 | 5.1 | 0.98 | 0.51 | 1.21 | 2.16 | Benz. 0.5% | 60 |
| 7 | 2.4 | 6.6 | 0.96 | 0.43 | 1.10 | 1.20 | Benz. 0.5% | 60 |
| 8 | 2.55 | 5.7 | 0.92 | 0.61 | 1.28 | 1.70 | Benz. 1.0% | 15 |
| | | 9.0 | | 1.32 | | 1.45 | | 15 |
| 9 | 1.8 | 6.0 | 0.30 | 0.50 | 2.67 | 1.85 | Benz. 1.0% | 60 |
| 10 | 2.2 | 6.9 | 0.45 | 0 40 | 1.50 | 1.56 | Benz. 1.0% | 30 |
| | | 15.0 | | 3.60 | | 1.25 | | 60 |
| 11 | 2.6 | 4.3 | 0.52 | 0.256 | 1.9 | 1.83 | Benz. 1.0% | 30 |
| 12 | 2.8 | 6.75 | 0.39 | 2.76 | 2.1 | 2.0 | Benz. 1.0% | 60 |

TABLE 4

*Excitability of frog heart*

| EXPERIMENT NUMBER | WEIGHT OF FROGS | RHEOBASE | CHRONAXIE | $Q = Vc/Vr$ | INJECTION OF BENZEDRINE SULFATE IN VENTRAL LYMPH SAC 60 MINUTES EARLIER |
|---|---|---|---|---|---|
| | *grams* | *volts* | *sigmas* | | *mgm.* |
| 1 | 160 | 6 | 7.2 | 1.03 | 60 |
| 2 | 165 | 4.9 | 8.8 | 1.03 | 40 |
| 3 | 155 | 7.2 | 6.8 | 1.03 | 38 |
| 4 | 130 | 6.3 | 7.2 | 1.05 | 32 |
| 5 | | 3.6 | 3.28 | 1.04 | Control |
| 6 | | 5 2 | 3.76 | 1.04 | Control |

D. *Action of benzedrine on the excitability of the heart.* Experiments were performed on animals poisoned by the injection of benzedrine in the ventral lymph sac prior to the tests. In table 4 it can be seen that the rheobase increased, the chronaxie was doubled, and the accommodation was unchanged (constant value of $Q$). This indicates that benzedrine induces a state of hypo-excitability of the cardiac muscle in the frog.

## CONCLUSIONS

1. Injection of benzedrine sulfate (150–600 mgm. per kgm.) in the ventral lymph sac of the frog causes, after one hour, a great increase in the chronaxie of the sciatic-gastrocnemius preparation and a reduction in the rate of nervous accommodation.
2. If the nerve is immersed in a physiologic solution of benzedrine (0.1–0.5–1.0%) there is a progressive increase in the galvanic threshold (rheobase) to the point of inexcitability of the preparation, with maintenance of the chronaxie, which is in agreement with the observations of Meidinger.
3. Under the action of benzedrine sulfate the gastrocnemius muscle shows a considerable increase in the rheobase; at the same time the chronaxie decreases by half when a dilute solution is used. With a more concentrated solution a twofold effect is observed, first a reduction in the chronaxie and later an increase. The voltage quotient varies inversely with the chronaxie.
4. In frogs poisoned by benzedrine the heart shows an increase in the rheobase as well as in the chronaxie, with no change in the rate of accommodation of the cardiac muscle.
5. In every instance benzedrine sulfate causes a decrease in excitability through an exaggerated increase in the rheobase or the chronaxie, an effect which may be preceded by a brief period of increased excitability.

The author wishes to thank Dr. H. Kallas, Director of the Institute of Physiology at the University of Concepción, for his interest in this work and for his valuable criticism.

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# TOXICITY OF BENZEDRINE SULFATE IN THE WHITE MOUSE AND IN THE FROG (*CALYPTOCEPHALUS GAYI*)

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Considerable disagreement exists concerning the toxic dose of benzedrine in animals. This is particularly evident with respect to subcutaneous injection in white mice. According to Marri and Franceschini (1) the $LD_{50}$ is 280 mgm. per kgm., according to Chakravarti (2) it is 155 mgm. per kgm., and according to Halpern (3) it is 25 mgm. per kgm. The subcutaneous lethal dose for white mice seemed therefore to warrant further investigation. Inasmuch as the literature contains no data on the lethal dose in frogs, this was also included.

RESULTS. *Mice.* Toxicity in relation to the time elapsing between the injection of benzedrine and the death of the animal was first determined. As is apparent from table 1, the latent period increased in inverse ratio to the dose injected. The smallest dose given in this series was 266 mgm. per kgm., which was not lethal.

In a second series of experiments the $LD_{50}$ in white mice was determined. Doses of benzedrine ranging from 250 to 300 mgm. per kgm. were injected. Six female mice were used for each dose. In table 2 the animals which died are marked with a †. Young animals showed no greater resistance than older ones. The $LD_{50}$ for white mice was 270 mgm. per kgm., as deduced from the mortality calculated according to Behrens, quoted by Burn (4). The ratio $LD_{100}/LD_0$ gives a quotient of 1.16 which, according to Burn, would indicate great accuracy of the method used.

*Frogs.* Benzedrine in Ringer's solution was injected into the ventral lymph sac of the Chilean frog (*Calyptocephalus Gayi*). It is noteworthy that, whereas in white mice benzedrine poisoning regularly produced signs of hyperexcitability of the nervous system (agitation, tremor, clonus, motor incoordination), the frogs reacted at once with marked diminution of excitability. This also was observed by Meidinger (5) with doses above 750 mgm. per kgm. Boyd (6), *moreover, found that benzedrine increased reflex time of decerebrate frogs* by as much as 172%. The various reflexes were always abolished in a certain definite sequence, first the postural reflexes, then the corneal reflex and the reflex of the anterior extremities.

To estimate the $LD_{50}$ in the frog, three animals were injected with doses ranging from 250 to 300 mgm. per kgm. It was often difficult to determine the exact time of the animal's death since the reflexes decreased very gradually to the point of disappearance. In some instances the reflexes had almost entirely disappeared, yet the animal was still able to make slow voluntary movements for as much as four days after the injection. The $LD_{50}$ was found to be 280 mgm. per kgm., and the ratio $LD_{100}/LD_0$ was 1.2.

TABLE 1

*Subcutaneous injection of benzedrine sulfate, 1% in distilled water, male white mice*

| | EXPERIMENT NUMBER | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Weight (*grams*)...... | 25 | 25 | 10 | 13 | 12 | 12 | 15 | 15 | 18 | 18 | 17 | 15 |
| Dose (*mgm. per kgm.*)............. | 800 | 400 | 400 | 380 | 330 | 410 | 330 | 330 | 280 | 280 | 290 | 266 |
| Latent period before death (*minutes*).... | 16 | 27 | 27 | 43 | 43 | 58 | 70 | 74 | 125 | 130 | 132 | |

TABLE 2

*Subcutaneous injection of benzedrine sulfate, 1% in distilled water, in female white mice*

Twenty-four-hour observation period

| | DOSE (MGM. PER KGM.) | | | | | |
|---|---|---|---|---|---|---|
| | 250 | 260 | 270 | 280 | 290 | 300 |
| Weight (*grams*) | 20<br>18<br>16<br>17<br>14<br>14 | 11†<br>14†<br>15<br>9<br>7<br>13 | 14<br>10<br>8†<br>12†<br>9†<br>9 | 20<br>7†<br>12†<br>18†<br>11<br>14† | 12†<br>9†<br>15†<br>8†<br>7†<br>24† | 11†<br>18†<br>8†<br>11†<br>16†<br>16† |
| Mortality found | 0/6 | 2/6 | 3/6 | 4/6 | 6/6 | 6/6 |
| Mortality calculated (Behrens) | | 2/11 | 5/10 | 9/11 | | |
| Lethal dose | $LD_0$ | | $LD_{50}$ | | $LD_{100}$ | |

† Death

TABLE 3

*Injection of benzedrine sulfate, 1% in Ringer's solution, in the ventral lymph sac of the frog*

Twenty-four-hour observation period

| | DOSE (MGM. PER KGM.) | | | | | |
|---|---|---|---|---|---|---|
| | 250 | 260 | 270 | 280 | 290 | 300 |
| Weight (*grams*) | 146<br>172<br>167 | 133<br>160<br>182† | 210†<br>111<br>148 | 260†<br>157<br>180† | 182†<br>193<br>162 | 282†<br>200†<br>142† |
| Mortality found | 0/3 | 1/3 | 1/3 | 2/3 | 1/3 | 3/3 |
| Mortality calculated (Behrens) | | 1/8 | 2/7 | 4/7 | 5/7 | |
| Lethal dose | $LD_0$ | | | $LD_{50}$ | | $LD_{100}$ |

† Death.

## SUMMARY

(1) In white mice benzedrine poisoning produced signs of nervous hyperexcitability, while in frogs there was immediate diminution and eventual disappearance of all reflexes in a certain definite sequence.

(2) The $LD_{50}$ of benzedrine sulfate in white mice was 270 mgm. per kgm. by subcutaneous injection. Injected into the ventral lymph sac of frogs, it was 280 mgm. per kgm.

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# EFFECTS OF EPINEPHRINE AND PITRESSIN ON THE CORONARY ARTERY INFLOW IN ANESTHETIZED DOGS[1, 2]

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Considerable controversy exists in the literature in regard to the effect of epinephrine on the coronary blood flow. Barbour and Prince (2) and Eliot (3) found that *epinephrine* increased the mean inflow in saline-perfused, beating hearts of rabbits and cats. Anrep and Stacey (4), Markwalder and Starling (5) and Stoland and Ginsberg (6) also reported that it increased the mean coronary sinus outflow in anesthetized dogs and in heart-lung preparations. However, Barbour and Prince (2) and Kountz (7) found that it decreased the mean inflow in saline-perfused monkey and human hearts, and Drury and Sumbal (8) that it decreased the coronary sinus outflow from saline-perfused tortoise hearts. Brodie and Cullis (9) observed that, in saline-perfused rabbit hearts, epinephrine in small doses decreased, but in large doses increased the mean inflow and mean coronary sinus outflow. They found, however, that with the larger doses the increased flow was usually preceded by an interval of decreased flow which either preceded or coincided with the increased vigor of contraction of the ventricle. On the other hand, Gruber and Roberts (10) found that in beating cat, rabbit and rat hearts, perfused with mixture of saline and blood, the mean coronary sinus outflow increased with small doses but frequently decreased with larger doses of epinephrine, especially when the pure alkaloid was used. Essex Wégria, Herrick and Mann (11) found that the mean inflow recorded with the thermostromuhr was increased one and one-half to four times in the unanesthetized dog. Wegria, Essex, Herrick and Mann (12) reported that in the anesthetized dog the flow appeared to be increased relatively more than the mean pressure. Epinephrine has usually been found to decrease the rate of flow in hearts which had ceased to beat spontaneously during the course of an experiment (2, 10, 13), in hearts stopped by perfusion with unoxygenated sodium chloride solution (14), and in human hearts stopped by alkaline solution (15). However, the flow was increased by epinephrine in hearts stopped by removal of calcium or by perfusion of acid solution (7), (15), after administration of ergotoxin (13), in quiescent ventricular strips (3) and usually in fibrillating cat, rabbit and especially dog hearts (16, 17, 18). In instantaneous flow records obtained with the hot wire meter on perfused cat, rabbit and dog hearts and on heart-lung preparations Rössler and Pascual (17) and Hausler (19) found that

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[1] A preliminary report of this work was presented at the meeting of the American Physiological Society, 1939 (1).

[2] Supported by a grant from the Council on Pharmacy and Chemistry of the American Medical Association.

epineprhine decreased systolic inflow, and sometimes caused systolic backflow. However, it increased the diastolic inflow later, but to a less marked extent than the nitrites. In records taken with the phototachometer Kliesecki and Flek (20) observed that epinephrine increased the diastolic flow apparently less than would be expected from the rise of aortic pressure.

All observations agreed that *pitressin* decreased the mean and the diastolic inflow in saline-and in blood-perfused beating rabbit, dog, human, cat and rat hearts (7, 13, 15, 19, 21, 22, 23, 24) and that it decreased the mean coronary sinus outflow in anesthetized dogs, heart-lung preparations and saline perfused rabbit hearts (3, 25, 26, 27, 28, 29). Pitressin decreased the mean inflow in saline-perfused quiescent and fibrillating hearts of sheep, dogs, cats and rabbits (13, 18, 22, 30), although an increased inflow in the perfused beating cat heart was reported (23). Essex, Wégria, Herrick, and Mann (11, 12) reported that the mean coronary artery inflow, measured by the thermostromuhr in both anesthetized and unanesthetized dogs, was reduced despite increased blood pressure.

The divergence of results following administration of epinephrine appears to be due in part to use of inaccurate recording devices, to failure of mean flow methods to differentiate dynamic extravascular effects from direct effects upon the vessels, to the fact that almost all of the flow measurements have been made on perfused hearts or heart-lung preparations under somewhat abnormal dynamic, chemical or nervous conditions, and perhaps in part to the hydrogen ion concentration of the drug solutions, and to the effect of the preservatives added to the commercial products. As a result, it is not possible to state definitely what are the effects of epineprhine upon the coronary circulation in the whole animal.

The development of a flow meter by Gregg and Green (31) by means of which the phasic coronary flow may be recorded continuously along with optical records of the pressure pulses has made possible the detailed analysis of the effects of epineprhine on the coronary circulation in the whole animal. These data are presented in this paper. The observations in the literature regarding the effects of pitressin are less controversial. Data on the effects of this drug on the coronary circulation as measured with the orifice meter are also presented.

*Methods.* Twenty dogs averaging 15 Kg. were anesthetized with initial injections of morphine (40 mg.) subcutaneously and either sodium barbital (175 to 225 mg. per kg.) or chloralosane (55 to 65 mg. per kg ) intravenously. The chest was opened, usually by resection of the anterior part of the fourth and fifth left ribs, and the heart suspended in a pericardial cradle. The animal's blood was prevented from clotting by an initial intravenous injection of 0.07 to 0.1 cc. of heparin[3] plus 120 to 160 mg Calcomine fast pink[4] per kilogram.

In all experiments inflow was measured in the descending limb of the left coronary artery (see figure 1) by leading the blood from the aorta (*A*) through the orifice meter (*O*) into the coronary artery (*C*), thus metering the flow while the coronary artery was supplied by a pulsatile stream from the animal's own aorta. In some additional experiments the coronary artery was perfused with blood under a known constant head of pressure from a reservoir connected to $S^1$.

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[3] Liquaemine—courtesy of the Roche-Organon Co.

[4] Courtesy the Calco Chemical Co.

The blood was obtained from the aorta by a large cannula inserted into the ascending aorta by way of the carotid artery. The blood entered the coronary artery either by way of a cannula inserted into a side branch, the main ramus being clamped proximally during flow measurements, or by way of a cannula inserted in the main ramus as shown in figure 1.

The orifice meter (31) (see figure 1) consists of two tubes of about 2.4 mm. bore separated by a thin metal plate (*O*) which had an orifice about 1 mm. in diameter. An optically recording differential manometer (*DM*) was connected to these tubes so as to record the difference between the lateral pressure proximal to and that just distal to the orifice. In the absence of flow, the upstream and downstream pressures are the same. When flow occurs the down stream pressure is lowered with respect to the upstream pressure by a small amount which varies roughly with the square of the rate of flow and which causes a corresponding deflection of the recording beam of the differential manometer. The meter was calibrated frequently during an experiment by turning the stopcocks $S^1$ and $S^2$ so as to shunt the coronary flow through the tube *SH* and then running blood at known rates of flow through the meter by way of stopcocks $S^3$ and $S^4$ while recording the deflections of the light beam.

In interpreting the flow records obtained during an experiment the rate of flow (in cc. per min.) at any instant was determined by comparing the deflection at that instant with the deflections obtained during the above-mentioned calibrations, which are indicated on the figures by the scale at the left of the flow curves. The total flow in cc. over any interval of time (say a single systole) was ascertained by integration after redrawing the recorded flow curves with a linear ordinate scale (see reference (33)). In the curves illustrated in the figures, the flow in cc. for systole was multiplied by the heart rate to give the systolic flow in cc. per minute (*S*). The same computation was made for the diastolic flow (*D*). The sum of these is the total or mean flow in cc. per minute (*T*).

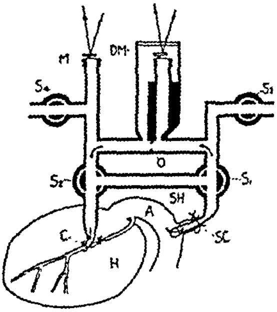

FIG. 1. Diagram of the connections of the optically recording orifice meter and pressure manometer. *H*—heart; *C*—ramus descendens of the left coronary artery; *A*—aorta; $S_{1,2,3,4}$—stopcocks for controlling the direction of blood flow during recording of the coronary flow and during calibration; *SH*—shunt for coronary flow during calibration of the meter; *O*—orifice; *DM*—differential manometer; *M*—pressure manometer. Arrows indicate paths of the light beams used in optical registration.

The pulsatile or constant pressure at which the coronary vessels were perfused was optically recorded by a rubber or a glass (34) membrane manometer of adequate frequency (*M*—figure 1). In some experiments an optical myograph (35) recorded the amplitude of the systolic shortening in the ventricular area, the coronary flow of which was being measured. All manometers were mounted on a rigid optical manometer stand (32).

RESULTS. The characteristics of the normal coronary inflow may conveniently be pointed out in the lowest curve (*F*) in the first segment of figure 2. At the end of diastole (*A*) the rate of flow was rapid, 9.5 cc. per minute, but with

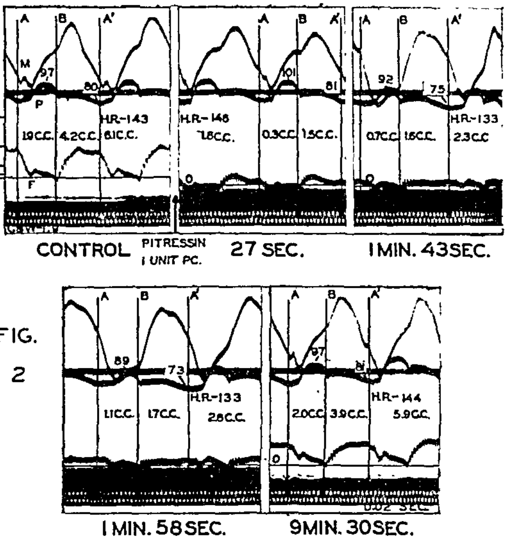

FIG. 2

FIG. 3

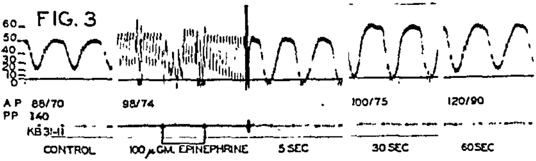

Fig. 2. Effects of an intracoronary injection of one pressor unit of pitressin dissolved in 2 cc of blood First segment—control Subsequent segments taken at the times indicated after the injection. Coronary artery perfused with blood under pulsatile head of [illegible] sys[illegible] of [illegible] mn[illegible] in [illegible] the [illegible] flo[illegible] GBW1-9.

Fig. 3. [illegible] 000 solution dissolved in 2 cc. blood) injected [illegible] with blood under constant head of pressure [illegible]. Scale at left—rate of flow in cc. per minu[illegible]t $\frac{1}{50}$ second. Kymograph operated at about [illegible] the onset of isometric contraction (*A*) the flow diminished abruptly due to the extravascular compressing effect of the contracting cardiac muscle. The rate of flow increased during the rise of aortic pressure accompanying ventricular ejection, decreased slightly in the latter half of systole (to 1.6 cc. per min, at B), increased rapidly during the isometric relaxation of the ventricle beginning shortly after B, and declined gradually during the latter part of diastole as aortic pressure fell.

PITRESSIN. The effects of 0.1 to 2.0 units of pitressin were studied following 13 injections in 9 dogs. The results were essentially the same in all tests. The typical effects of an intracoronary injection of 1 unit of pitressin are illustrated in figure 2. Within 27 seconds after the injection the flow at the end of diastole (*A*) was reduced to 2.8 cc. per minute, and at the end of systole (*B*) to 0.35 cc. per minute, despite a slight elevation of the corresponding aortic pressures. The total systolic flow was similarly reduced from 1.9 to 0.3 cc. per minute and the total diastolic flow from 4.2 to 1.5 cc. per minute, thus accounting for the reduction of the mean flow to 30% of the control (from 6.1 to 1.8 cc. per minute). After 1 min. 43 sec., the flow was still reduced.

In the first two segments of figure 2 shortening of the muscle, indicated by upward deflection of the myogram (*M*), began with ejection. One minute and 43 seconds after the injection of pitressin, however, the muscle lengthened abruptly with the onset of isometric contraction (*A*) and began to shorten only as intraventricular pressure fell at the onset of diastole (*B*). In other words, instead of shortening during systole, the perfused area was being stretched by the force of the rising intraventricular pressure. At this time the heart rate and blood pressure had decreased slightly. In the last segment of figure 2, taken nine minutes and 30 seconds after the pitressin injection, the blood pressure, heart rate, coronary flow and myogram had returned approximately to control levels.

EPINEPHRINE. Epinephrine was studied by 26 intracoronary and 3 intravenous injections (figs. 4–7) of commercial preparations—1:1,000 solution containing chloretone—in 14 dogs and by 7 intracoronary injection of the alkaloid,[5] dissolved in N/10 HCl in physiological saline in 4 dogs. All intracoronary injections consisted of the drug dissolved in a minimum quantity of solvent (0.1 to 0.01 cc.) diluted in 2 cc. of blood. This was done in order to eliminate the temporary augmentation of coronary flow due to the lowering of viscosity caused by injections of larger quantities of saline (36). Eight control injections in 5 dogs of 2 cc. of blood containing a similar quantity of saline had no effect upon coronary flow.

In all cases, injection of epinephrine caused a typical series of changes such as are illustrated in the curves reproduced in fig. 4. To facilitate analysis, the flow and pressure curves have been traced and the flow curves redrawn with a linear ordinate scale. One hundred μgms. of epineprhine were injected intravenously at the arrow ↑. Coincident with the rise of aortic pressure—second and third segments—the mean coronary flow (*T*) increased from 17.2 to 31.4

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[5] Courtesy of Parke, Davis and Co.

cc./min. The increased mean flow was due to the augmentation of diastolic flow (*D*) from 11.6 to 27.0 cc./min., due principally to the greater inflow in middle and late diastole. The systolic inflow (*S*) was reduced from 5.6 to 4.4 cc./min. due to the shortening of the systole cycle ratio, to the appearance of backflow during isometric contraction, and to the persistence of a low inflow rate during the ejection phase of systole—despite the elevation of aortic systolic pressure. The backflow during isometric contraction was to some extent compensated by an accelerated inflow during the isometric relaxation phase of diastole (just following (*B*)).

*A. Comparison of the mean rate of inflow with the mean aortic pressure.* In order to estimate whether the increase of coronary flow was greater or less than that anticipated from the rise of aortic pressure, i.e., whether coronary constriction or dilation occurred, we have compared the increase of coronary flow induced by raising the aortic pressure by compression of the thoracic aorta (Fig. 5) with that occurring during the rise induced by the drug (Figs. 4 and 6). Comparison of the mean flow (*T*) with the mean aortic pressure—average of systolic and diastolic values—indicates that the ratio of flow to pressure increased slightly from 0.183 to around 0.23—during the effect of the epinephrine. When a similar dose of epinephrine was injected into the coronary artery (Fig. 5—solid lines), the mean flow increased considerably more than the corresponding mean aortic pressure so that the flow to pressure ratio changed from 0.189 to 0.288. On the other hand, when the aortic pressure was elevated by occlusion of the thoracic aorta (Fig. 6—solid line), the mean coronary flow increased, but the increase was relatively less than that of the mean aortic pressure, the ratio of flow and pressure dropping from 0.202 in the control to 0.180 during compression of the aorta.

*B. Coronary flow at the end of diastole (diastolic conductance—D.C.).* Comparisons of the mean flow with the mean aortic pressure suggest that epinephrine may have a coronary dilator action as reported by Wégria, Essex, Herrick and Mann (12). The increase in mean flow might, however, be due to changing mechanical factors such as changing *S/C* ratio which, as may be seen from columns *S/C* and *E/C* of table 1, follow epineprhine injections. In order to determine what part of the response was due to a coronary dilation we have calculated the ratio of the instantaneous rate of flow at the end of diastole to the simultaneously existing aortic pressure. This ratio will be called the *diastolic conductance.* This instant in the heart cycle was chosen since it is least complicated by extraneous factors such as the extravascular compression and refilling of the vessels present during systole and the first half of diastole respectively (37). A tabulation of this data for a few typical experiments is presented in column *DC* of tables 1 and 2.

Such comparison for the records presented in figures 4–6 (*GW*14—5, 3 and 7) shows that with the intravenous epineprhine (fig. 4) the diastolic conductance increased from a control value of 0.30 to 0.32 at 33 sec., to 0.34 at 55 sec., and returned to 0.30 at 1 min. 32 sec. After the intracoronary injection the diastolic conductance increased from a control value of 0.22 to 0.46 at the peak of the

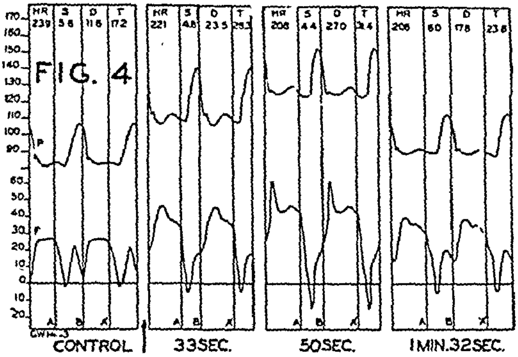
FIG. 4
HR 239 S 5.6 D 11.6 T 17.2
HR 221 S 4.8 D 23.5 T 28.3
HR 206 S 4.4 D 27.0 T 31.4
HR 206 S 6.0 D 17.8 T 23.8
P
F
A B A'
GW14-3
CONTROL
33SEC.
50SEC.
1MIN.32SEC.

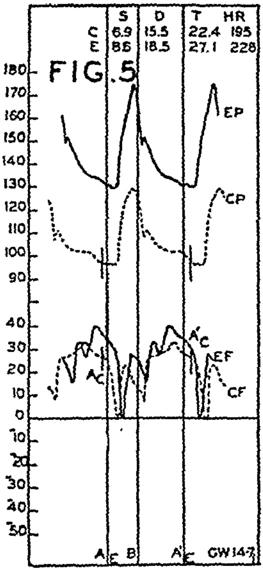
FIG. 5
S D T HR
C 6.9 15.5 22.4 195
E 8.6 18.5 27.1 228
EP
CP
EF
CF
A_C
A'_C
A_E B A'_E
GW14-7

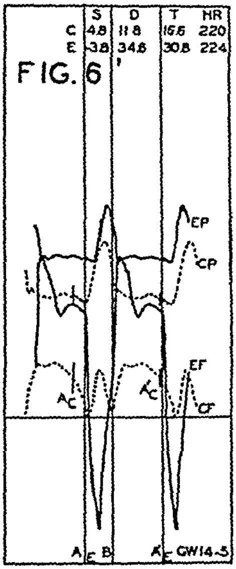
FIG. 6
S D T HR
C 4.8 11.8 16.6 220
E -3.8 34.6 30.8 224
EP
CP
EF
CF
A_C
A'_C
A_E B A'_E
GW14-5

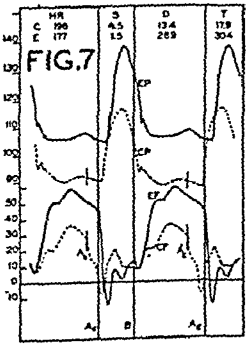
FIG. 7
HR S D T
C 196 4.5 13.4 17.9
E 177 1.5 28.9 30.4
EP
CP
EF
CF
A_E B A'_E

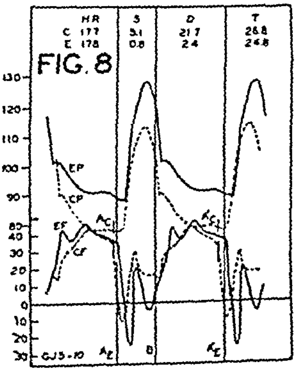
FIG. 8
HR S D T
C 177 5.1 21.7 26.8
E 178 0.8 2.4 24.8
EP
CP
EF
CF
A_C
A'_C
A_E B A'_E
GJ5-10

Figs. 4–8

effect. On the other hand, the diastolic conductance was essentially unchanged by elevation of aortic pressure produced by compression of the aorta.

The maximum increase in diastolic flow always occurred during the maximum of aortic diastolic pressure. However, the flow did not always return as rapidly to control levels as the pressure, with the result that diastolic conductance was often greater during the period of declining pressure than during the interval of maximum aortic pressure.

*C. The systolic coronary flow.* In addition to the effect on diastolic flow induced by epinephrine, one of the most marked phenomena was the augmentation of the reduction of inflow and the appearance of or increase in the backflow during the interval of isometric contraction beginning at *A* or $A^1$ in the illustrations. The minimum inflow during the period (or the maximum backflow) tabulated in column *MSF* in table 1 indicates the magnitude of the effect. The inflow was also often decreased by the epineprhine during the systolic peak of the aortic pressure (*PSF* in table 1). However, the inflow was frequently increased at the end of systole (*B* in the illustrations—*ESF* in table 1).

*D. Perfusion under a constant head of pressure.* In the above experiments the coronary artery was perfused with blood from the aorta. As a result, the perfusion pressure was increased by the drug about the same time that changes in flow, due to direct effects of the drug on the vessels, was to be expected. In order to eliminate this variable, the coronary artery was perfused with blood from a reservoir, maintained at a constant pressure by an air system, 13 times in 4 animals. The data from these experiments, presented in table 2, indicate that the effects on diastolic conductance were essentially the same as when perfused from the aorta. As was to be expected, however, the reduction of inflow or the magnitude of backflow, at the instant corresponding to the peak of systolic aortic pressure, was much greater with the constant pressure perfusion.

---

[illegible] heart cycle (mea[illegible]

time following injection—other letter-

Fig. 6. Effect of aortic compression. Same experiment as fig. 4 (GW14-7)—coronary artery perfused with blood under pulsatile head of pressure from aorta. Dashed lines *CP*, *CF*—control; solid lines, *EP*, *EF*—effect of experimental procedure on aortic pressure (*CP*, *EP*) and coronary flow (*CF*, *EF*) respectively. Control and experimental curves [illegible] control and [illegible] Hg. Lower [illegible] cc. of 1:1000 [illegible] W14-5). For lettering see figure 5.

Fig. 7. Effects of 10 μ gm. commercial epinephrine (1:1000 solution diluted in 2 cc. blood). For lettering see figure 5.

Fig. 8. Effect of 10 μ gm. "pure" epinephrine alkaloid dissolved in minimal quantity dilute HCl and diluted in 2 cc. blood Same experiment as figure 7. For lettering see figure 5.

TABLE I

*Tabulation of data from four typical experiments out of 15 in which the coronary artery was perfused with blood from the aorta, i.e., under a pulsatile head of pressure*

| NO. | DRUG | DOSE | AP AND PP | | MSF | | PSF | | ESF | | DF | | DC | | HR | | S/C | | E/C | | DC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C | E | C | E | C | E | C | E | C | E | C | E | C | E | C | E | C | E | % CONTROL |
| | | μgm. | | | | | | | | | | | | | | | | | | | |
| GJ6-6 | C | 1 | 110/81 | 119/85 | −14 | −29 | 10 | 5 | 5 | 8.5 | 22 | 26 | 0.27 | 0.31 | 176 | 176 | 0.42 | 0.38 | 0.35 | 0.27 | 115 |
| GJ6-2 | C | 10 | 90/63 | 95/65 | 0 | −23 | 8.5 | 8.5 | 13 | 21 | 37 | 43 | 0.54 | 0.66 | 193 | 191 | 0.43 | 0.34 | 0.32 | 0.25 | 122 |
| GJ6-1 | C | 100 | 104/74 | 175/120 | 5 | | 13 | 40 | 10 | 37 | 33 | 88 | 0.45 | 0.73 | 193 | 200 | 0.42 | 0.37 | 0.35 | 0.30 | 162 |
| GJ6-7 | P | 1 | 104/75 | 113/80 | −19 | −26 | 0 | 0 | 3.5 | 2 | 18 | 17 | 0.23 | 0.21 | 176 | 176 | 0.46 | 0.44 | 0.35 | 0.26 | 91 |
| GJ6-5 | P | 10 | 94/70 | 115/83 | − 5 | −34 | 5 | 4 | 8.5 | 14 | 24 | 28 | 0.34 | 0.34 | 188 | 185 | 0.44 | 0.37 | 0.31 | 0.23 | 100 |
| GJ5-5 | C | 10 | 118/92 | 138/106 | − 8 | −14 | 9 | 2 | 8 | 13 | 27 | 45 | 0.29 | 0.43 | 196 | 177 | 0.46 | 0.33 | 0.31 | 0.24 | 148 |
| GJ5-8 | P | 10 | 96/76 | 105/86 | −12 | −38 | 18 | 0 | 12 | 4 | 22 | 34 | 0.29 | 0.40 | 187 | 188 | 0.37 | 0.41 | 0.25 | 0.28 | 138 |
| GJ5-10 | P | 10 | 125/87 | 131/93 | −15 | −28 | 21 | 0 | 18 | 11 | 37 | 37 | 0.43 | 0.40 | 177 | 178 | 0.38 | 0.36 | 0.28 | 0.27 | 93 |
| GW14-5 | C | 100 | 106/82 | 122/98 | 0 | −48 | 10 | 0 | 3 | 16 | 18 | 45 | 0.22 | 0.46 | 220 | 224 | 0.45 | 0.34 | 0.31 | 0.22 | 196 |
| GW14-3 | IV | 100 | 106/83 | 153/126 | − 2 | −14 | 13 | 17 | 5 | 24 | 25 | 43 | 0.30 | 0.34 | 239 | 208 | 0.52 | 0.41 | 0.30 | 0.20 | 113 |
| GW14-7 | Comp. aorta | | 129/98 | 175/130 | 0 | 0 | 15 | 26 | 13 | 25 | 26 | 34 | 0.27 | 0.26 | 212 | 208 | 0.40 | 0.40 | 0.26 | 0.24 | 96 |
| GD16-6 | C | 1 | 89/65 | 90/67 | − 0.9 | − 4.8 | 0.5 | 0 | 0 | 0 | 2.4 | 2.8 | 0.037 | 0.042 | 169 | 171 | 0.40 | 0.38 | 0.30 | 0.24 | 113 |
| GD16-3 | C | 10 | 103/81 | 107/81 | 0 | − 5.2 | 2 | 0 | 0 | 0 | 4.2 | 6.0 | 0.052 | 0.074 | 190 | 188 | 0.45 | 0.36 | 0.24 | 0.21 | 142 |
| GD16-4 | C | 100 | 106/85 | 113/89 | − 0.8 | − 6.4 | 0.8 | −2 | 0 | 0 | 3.7 | 5.7 | 0.044 | 0.064 | 185 | 187 | 0.43 | 0.40 | 0.25 | 0.23 | 145 |
| GD16-8 | IV | 5 | 110/85 | 124/92 | − 0.5 | − 1.3 | 1.6 | 0.8 | 0.8 | 0.5 | 2.2 | 2.4 | 0.026 | 0.026 | 154 | 150 | 0.40 | 0.43 | 0.25 | 0.28 | 100 |

*No.*, animal and test number. Dose, μ gms. of epinephrine. *P*, "pure" alkaloid obtained from Dr. McGinty of the Parke, Davis Co. *C*, commercial 1:1000 adrenalin. *Comp. aorta*, effect of raising aortic pressure by compressing the thoracic aorta. *AP* and *PP*, aortic pressure, in mm. Hg, *systolic (SP)/diastolic (DP)*, *perfusion pressure (PP)* equals aortic pressure. *C*, control; *E*, data during maximum effect of the drug. *MSF*, flow (in cc. per min.) at end of isometric contraction. *PSF*, flow at peak of systolic pressure; *ESF*, flow at end of systole (*B* in figures). *DF*, flow at end of diastole (*A* or *A′* in figures). A minus sign indicates backflow. *DC*, diastolic conductance = *DF*/*DP*. *HR*, heart rate in beats per minute. *S/C*, systole-cycle ratio. *E/C*, ejection-cycle ratio. *DC*% of control, diastolic conductance during the maximum effect of the drug (*E*) divided by the control diastolic conductance (C) × 100. IV, drug administered intravenously.

*E. The effect of the solvent.* Coronary dilation with the 1:1,000 solution has been said to be due to the presence of the chloretone or the altered pH due to the HCl. As used in these experiments, the HCl appeared to be ineffective since in 2 injections in 2 dogs of an amount equal to that in 0.1 cc. of the 1:1000 solution used, there was no effect on coronary flow. Injection of 0.01 cc. of commercial solvent[6] however, caused a small increase in coronary diastolic flow, but the effect was less than that caused by 0.01 cc. of the 1:1000 epinephrine solution. This solvent contained HCl and chloretone but no epinephrine.

TABLE 2

*Tabulation of data from all experiments in which the coronary artery was perfused with blood from a reservoir under an approximately constant head of pressure*

Lettering as for table I. In addition, *A* = commercial adrenalin (Parke, Davis Co.) alkaloid powder—ampules gr. I. *Com. Solv.* = 100 cc. of the solvent used in preparing Parke, Davis Co. 1:1000 adrenalin solution.

| NO. | DRUG | DOSE | PP | | PSF | | DF | | DC | | AP | | DC PER CENT CONTROL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C | E | C | E | C | E | C | E | C | E | |
| | | *mgm* | | | | | | | | | | | |
| GJ6-6 | C | 1 | 87 | 90 | 6 | 0 | 28 | 28 | 0.32 | 0.31 | 110/81 | 119/85 | 97 |
| GJ6-2 | C | 10 | 65 | 60 | 0 | −30 | 41 | 43 | 0.63 | 0.72 | 90/63 | 95/65 | 114 |
| GJ6-1 | C | 100 | 94 | 83 | 12 | −25 | 50 | 70 | 0.53 | 0.84 | 104/74 | 175/120 | 158 |
| GJ6-7 | P | 1 | 80 | 83 | 0 | 0 | 19 | 21 | 0.24 | 0.25 | 104/75 | 113/80 | 104 |
| GJ5-5 | C | 10 | 93 | 90 | 2 | −28 | 31 | 37 | 0.33 | 0.41 | 118/92 | 138/106 | 124 |
| GJ5-8 | P | 10 | 76 | 75 | 4 | −26 | 23 | 27 | 0.30 | 0.36 | 96/76 | 105/86 | 120 |
| GJ5-10 | P | 10 | 79 | 77 | 0 | −14 | 33 | 32 | 0.42 | 0.41 | 125/87 | 131/93 | 98 |
| KB31-10 | C | 10 | 123 | 117 | 15 | 0 | 29 | 44 | 0.24 | 0.38 | 81/63 | 95/65 | 158 |
| KB31-11 | C | 100 | 105 | 103 | 26 | 0 | 50 | 68 | 0.48 | 0.66 | 88/70 | 100/75 | 138 |
| KB31-12 | P | 10 | 122 | 105 | 19 | 0 | 32 | 43 | 0.26 | 0.41 | 70/50 | 80/65 | 158 |
| KB31-13 | P | 100 | 95 | 98 | 22 | 9 | 42 | 51 | 0.44 | 0.52 | 70/50 | 90/70 | 118 |
| KB85-7 | Com. solv. | | 94 | 92 | −5.5 | −12 | 41 | 44 | 0.44 | 0.48 | 115/92 | 113/85 | 109 |
| KB85-8 | A | 10 | 94 | 88 | −10 | −47 | 45 | 65 | 0.47 | 0.74 | 110/85 | 115/86 | 158 |
| KB85-9 | C | 10 | 94 | 90 | −5.5 | −47 | 43 | 63 | 0.46 | 0.70 | 107/85 | 110/78 | 152 |

Seven comparisons were made in 4 dogs of the effects of the injection of commercial 1:1000 solution, and injection of a similar amount of the alkaloid freshly dissolved in N/10 HCl. The results are presented in tables 1 and 2. Allowing for slight variability of dosage, the two caused quite comparable effects on the *S/C* ratio and on the inflow during systole. The commercial solution, however, caused in four trials a somewhat greater increase of diastolic flow and particularly of diastolic conductance. The maximum difference in the effects is illustrated in figures 7 and 8.

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[6] Courtesy of Parke, Davis and Co.

*F. The minimum effective dose.* Intracoronary injections varying from less than 1 to more than 100 μgm. were given. No effect was produced with less than 1 μgm. With increasing doses the effects on aortic pressure, diastolic inflow and systolic backflow, and $S/C$ ratio became more marked and in general the diastolic conductance was proportionally increased, as is shown by the columns labelled $DC$ per cent of control in tables 1 and 2.

*G. The time relation between increased vigor of contraction and increased diastolic conductance.* The smaller increase of conductance with intravenous injections and with the intracoronary injection of the pure alkaloid as compared with the more prominent effects on arterial pressure and on systolic inflow suggest that some of the increased inflow may be due to vasodilation induced by accumulation of metabolic products as a result of the direct stimulation of the myocardium. Figure 3, a record of the inflow under constant perfusion pressure, shows what we have constantly observed, that the reduction of systolic inflow precedes the augmentation of diastolic inflow.

*H. Antagonism between pitressin and epinephrine.* It has been reported by Melville and Stehle (38), and by Melville (39) that pitressin and epinephrine exert antagonistic effects. This we have confirmed. Administration of a 0.1 cc. of the 1:1000 solution during a period of reduced coronary flow induced by 1 or 2 units of pitressin caused an immediate increase of the vigor of contraction and acceleration of diastolic inflow in 2 tests in 2 dogs. Conversely, administration of 0.8 and 2 units of pitressin during a period of elevated aortic pressure induced by a continuous intravenous infusion of epinephrine caused an immediate reduction of the coronary inflow in 2 tests in 2 dogs.

DISCUSSION. Pitressin apparently causes primarily a constriction of the coronary vessles with more or less proportional reduction of inflow throughout the cycle. Epinephrine, however, apparently has a twofold effect. The first is to cause a marked reduction of inflow during early and midsystole and even the appearance of backflow during the isometric contraction phase. This phenomenon is apparently due to an increased vigor of myocardial contraction and a more effective extravascular compression of the coronary vessels by the contracting myocardium since it occurs prior to any change of aortic pressure or of diastolic inflow. The second effect is an increased inflow in diastole due in part to the rising aortic pressure, but due also to dilation of the coronary vessels. That the coronary vessels actively dilate is indicated by the increased diastolic conductance observed when the vessels were perfused from the aorta, as well as when they were perfused at a constant head of pressure. No evidence of a coronary constrictor effect resembling that of pitressin has been obtained. It seems probable that the apparent constriction obtained in beating hearts by mean flow methods may have been due to a reduction of systolic inflow by the greater extravascular compression exerted by the more vigorously contracting myocardium rather than to an active intrinsic constriction of the coronary vessels.

The coronary dilator action of the commercial 1:1000 solution of adrenalin was definite. It is probable, however, that some of this effect was due to the chloretone present in the commercial preparation, since the dilator action was less prominent with the "pure" alkaloid dissolved in HCl, and since the commercial solvent also caused coronary dilation. A small amount of coronary dilation, i.e., a smaller increase in diastolic conductance was, however, still seen with the "pure" alkaloid. This probably represented active dilation in response to the drug. Even this amount of dilation, however, may have been due to the augmented metabolic activity of the myocardium, since in these experiments the effect upon the contractile effort of the myocardium—measured by the reduction of systolic inflow—consistently preceded the effect on the diastolic coronary flow.

The considerable augmentation of mean coronary flow reported in the literature to follow epinephrine, an effect which seemed to be greater than the elevation of mean aortic pressure, may have been due, in addition to the above factors, to a relative shortening of systole, allowing more diastolic time per minute, and to a more rapid relaxation of the myocardium in early diastole. The latter is suggested by the more rapid rise of coronary flow in early diastole seen in these experiments and the more rapid decline of the peripheral coronary pressure in early diastole reported elsewhere (37). The marked augmentation of flow observed with the thermostromuhr may in addition be partly artifact due to the markedly increased oscillation of flow, to zero and below during systole, following epineprhine, an occurrence which as Gregg *et al.* (40, 41) have have shown, can lead to abnormally high flow readings with this instrument.

Graybiel and Glendy (42) report that angina rarely follows administration of pitressin, whereas it not infrequently follows administration of epinephrine. These observations might be interpreted to mean that in man these drugs have different effects than in the experimental animal. It is more probable, however, as Graybiel and Glendy suggest, that in addition to its coronary constrictor action pitressin may depress myocardial metabolism sufficiently so that a relative insufficiency of myocardial blood supply does not occur with ordinary clinical doses. The agina following epinephrine may be due to a relative insufficiency of the coronary circulation due to the metabolic demands of the myocardium exceeding the increase of coronary flow.

As may be seen from the records presented in this paper, epinephrine causes a more abrupt and vigorous contraction, and also increases the work of the heart by elevating the aortic pressure and probably the cardiac output. These changes must result in increasing the metabolic demands of the myocardium. Furthermore, according to studies of Gremmels (43), Gollwitzer-Meier and Kroetz (44) and Gauer and Kramer (45), it is possible that epinephrine may decrease the metabolic efficiency of the myocardium. As a result, the $O_2$ consumption per unit of work performed by the heart will be increased and thus the demands on the circulation will be further augmented.

Green and Wegria (32) have shown that the coronary vessels are readily dilated by metabolic products. It would seem therefore that this phenomenon plus a direct coronary dilation effect of the epinephrine should prevent the development of a relative insufficiency of the coronary circulation, despite the increased metabolic demands. Since angina does appear, the direct coronary dilator action of epinephrine must be relatively weaker than the effect on myocardial matabolism. In fact, it seems possible that the apparent coronary dilation following epinephrine is due entirely to an increased production of metabolic products.

## SUMMARY

Pitressin is an intense coronary constrictor capable, upon intracoronary injection, of causing sufficient constriction and reduction of coronary flow to depress the contractile effort of the myocardium.

Epinephrine is not a coronary constrictor in the dog. On the other hand, while the coronary flow increases after injection of the drug, this increase with the pure alkaloid is not much greater than would be anticipated from the rise of aortic pressure and follows by an interval of many seconds the appearance of myocardial stimulation induced by the drug. It is concluded therefore that epineprhine has only a weak coronary dilating effect *per se*, and that the apparently prominent coronary dilator action observed, particularly with the 1:1000 solution, is due in large part to a marked increase of myocardial metabolic activity, out of proportion to the increased cardiac work, to a coronary dilator effect of the preservative, and to a relative increase in the total diastolic time per minute due to shortening of the $S/C$ ratio.

Angina following epinephrine injection is probably due to failure of the drug to induce directly sufficient coronary dilation and increased blood flow, to supply the augmented myocardial metabolism, resulting in a relative myocardial ischemia.

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